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(54) Title: IκB KINASE, SUBUNITS THEREOF, AND METHODS OF USING SAME

(57) Abstract

The present invention provides an isolated nucleic acid molecule encoding IκB kinase (IKK) catalytic subunit polypeptides, which are associated with an IKK serine protein kinase that phosphorylates a protein (IκB) that inhibits the activity of the NF–κB transcription factor, vectors comprising such nucleic acid molecules and host cells containing such vectors. In addition, the invention provides nucleotide sequences that can bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful as probes or as antisense molecules. The invention also provides isolated IKK catalytic subunits, which can phosphorylate an IκB protein, and peptide portions of such IKK subunit. In addition, the invention provides anti–IKK antibodies, which specifically bind to an IKK complex or an IKK catalytic subunit, and IKK–binding fragments of such antibodies. The invention further provides methods of substantially purifying an IKK complex, methods of identifying an agent that can alter the association of an IKK complex or an IKK catalytic subunit with a second protein, and methods of identifying proteins that can interact with an IKK complex or an IKK catalytic subunit.

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IκB KINASE, SUBUNITS THEREOF, AND METHODS OF USING SAME

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to
molecular biology and biochemistry and more specifically
to a protein kinase, IκB kinase, which is activated in
response to environmental stresses and proinflammatory
signals to phosphorylate inhibitors of the NF-κB
transcription factors and to methods of using the protein
kinase.

BACKGROUND INFORMATION

The induction of gene expression due to
exposure of a cell to a specific stimulus is a tightly
controlled process. Depending on the inducing stimulus,
it can be critical to survival of the cell that one or
more genes be rapidly induced, such that the expressed
gene product can mediate its effect. For example, an
inflammatory response stimulated due to an injury to or
infection of a tissue results in rapid vasodilation in
the area of the injury and infiltration of effector cells
such as macrophages. Vasodilation occurs within minutes
of the response and is due, in part, to the expression of
cytokines in the injured region.

The rapid induction, for example, of an
inflammatory response or an immune response, requires
that the transcription factors involved in regulating

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such responses be present in the cell in a form that is
amenable to rapid activation. Thus, upon exposure to an
inducing stimulus, the response can occur quickly. If,
on the other hand, such transcription factors were not
5   already present in a cell in an inactive state, the
factors first would have to be synthesized upon exposure
to an inducing stimulus, greatly reducing the speed with
which a response such as an inflammatory response could
occur.

10   Regulation of the activity of transcription
factors involved in such rapid induction of gene
expression can occur by various mechanisms. For example,
in some cases, a transcription factor that exists in an
inactive state in a cell can be activated by a post-
15   translational modification such as phosphorylation on one
or more serine, threonine or tyrosine residues. In
addition, a transcription factor can be inactive due to
an association with a regulatory factor, which, upon
exposure to an inducing stimulus, is released from the
20   transcription factor, thereby activating the
transcription factor. Alternatively, an inactive
transcription factor may have to associate with a second
protein in order to have transcriptional activity.

25   Rarely, as in the case of glucocorticoids, the
inducing stimulus interacts directly with the inactive
transcription factor, rendering it active and resulting
in the induction of gene expression. More often,
however, an inducing stimulus initiates the induced
response by interacting with a specific receptor present
30   on the cell membrane or by entering the cell and
interacting with an intracellular protein. Furthermore,
the signal generally is transmitted along a pathway, for
example, from the cell membrane to the nucleus, due to a
series of interactions of proteins. Such signal
35   transduction pathways allow for the rapid transmission of

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an extracellular inducing stimulus such that the
appropriate gene expression is rapidly induced.

Although the existence of signal transduction
pathways has long been recognized and many of the
5   cellular factors involved in such pathways have been
described, the pathways responsible for the expression of
many critical responses, including the inflammatory
response and immune response, have not been completely
defined. For example, it is recognized that various
10 inducing stimuli such as bacteria or viruses activate
common arms of the immune and inflammatory responses.
However, differences in the gene products expressed also
are observed, indicating that these stimuli share certain
signal transduction pathways but also induce other
15 pathways unique to the inducing stimulus. Furthermore,
since inducing agents such as bacteria or viruses
initially stimulate different signal transduction
pathways, yet induce the expression of common genes, some
signal transduction pathways must converge at a point
20 such that the different pathways activate common
transcription factors.

A clearer understanding of the proteins
involved in such pathways can allow a description, for
example, of the mechanism of action of a drug that is
25 known to interfere with the expression of genes regulated
by a particular pathway, but the target of which is not
known. In addition, the understanding of such pathways
can allow the identification of a defect in the pathway
that is associated with a disease such as cancer. For
30 example, the altered expression of cell adhesion
molecules is associated with the ability of a cancer cell
to metastasize. However, the critical proteins involved
in the signal transduction pathway leading to expression
of cell adhesion molecules have not been identified.
35 Thus, a need exists to identify the proteins involved in

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signal transduction pathways, particularly those proteins
present at the convergence point of different initial
pathways that result in the induction, for example, of
gene products involved in the inflammatory and immune
5   responses. The present invention satisfies this need and
provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides isolated nucleic
acid molecules encoding full length human serine protein
10   kinases, designated IκB kinase (IKK) subunits IKKα and
IKKβ. The disclosed IKK subunits share substantial
sequence homology and are activated in response to
proinflammatory signals to phosphorylate proteins (IκB's)
that inhibit the activity of the NF-κB transcription
15   factor.

For example, the invention provides a nucleic
acid molecule having the nucleotide sequence shown as SEQ
ID NO: 1, which encodes a cytokine inducible IκB kinase
subunit designated IKKα, particularly the sequence shown
20   as nucleotides -35 to 92 in SEQ ID NO: 1, and nucleic
acid molecules encoding the amino acid sequence shown as
SEQ ID NO: 2, as well as nucleotide sequences
complementary thereto. In addition, the invention
provides a nucleic acid molecule having the nucleotide
25   sequence shown as SEQ ID NO: 14, which encodes a second
cytokine inducible IκB kinase subunit, designated IKKβ,
and nucleic acid molecules encoding the amino acid
sequence shown as SEQ ID NO: 15, as well nucleotide
sequences complementary thereto. The invention also
30   provides vectors comprising the nucleic acid molecules of
the invention and host cells containing such vectors.

In addition, the invention provides nucleotide
sequences that bind to a nucleic acid molecule of the

invention, including to nucleotides -35 to 92 as shown in
SEQ ID NO: 1. Such nucleotide sequences of the invention
are useful as probes, which can be used to identify the
presence of a nucleic acid molecule encoding an IKK
5   subunit in a sample, and as antisense molecules, which
can be used to inhibit the expression of a nucleic acid
molecule encoding an IKK subunit.

The present invention also provides isolated
full length human IKK subunits, which can phosphorylate
10   an IκB protein. For example, the invention provides an
IKKα polypeptide having the amino acid sequence shown as
SEQ ID NO: 2, particularly the amino acid sequence
comprising amino acids 1 to 31 at the N-terminus of the
polypeptide of SEQ ID NO: 2. In addition, the invention
15   provides an IKKβ polypeptide having the amino acid
sequence shown as SEQ ID NO: 15. The invention also
provides peptide portions of an IKK subunit, including,
for example, peptide portions comprising one or more
contiguous amino acids of the N-terminal amino acids
20   shown as residues 1 to 31 in SEQ ID NO: 2. A peptide
portion of an IKK subunit can comprise the kinase domain
of the IKK subunit or can comprise a peptide useful for
eliciting production of an antibody that specifically
binds to an IκB kinase or to the IKK subunit.
25   Accordingly, the invention also provides anti-IKK
antibodies that specifically bind to an IKK complex
comprising an IKK subunit, particularly to the IKK
subunit, for example, to an epitope comprising at least
one of the amino acids shown as residues 1 to 31 of SEQ
30   ID NO: 2, and also provides IKK subunit-binding fragments
of such antibodies. In addition, the invention provides
cell lines producing anti-IKK antibodies or IKK-binding
fragments thereof.

The invention also provides isolated IκB kinase
35   complexes. As disclosed herein, an IKK complex can have

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an apparent molecular mass of about 900 kDa or about 300
kDa. An IKK complex is characterized, in part, in that
it comprises an IKKα subunit, an IKKβ subunit, or both
and can phosphorylate an IκB protein.

5   The present invention further provides methods
for isolating an IKK complex or an IKK subunit, as well
as methods of identifying an agent that can alter the
association of an IKK complex or an IKK subunit with a
second protein that associates with the IKK *in vitro* or
10 *in vivo*. Such a second protein can be, for example,
another IKK subunit; an IκB protein, which is a substrate
for IKK activity and is involved in a signal transduction
pathway that results in the regulated expression of a
gene; a protein that is upstream of the IκB kinase in a
15 signal transduction pathway and regulates IKK activity;
or a protein that acts as a regulatory subunit of the IκB
kinase or of an IKK subunit and is necessary for full
activation of the IKK complex. An agent that alters the
association of an IKK subunit with a second protein can
20 be, for example, a peptide, a polypeptide, a
peptidomimetic or a small organic molecule. Such agents
can be useful for modulating the level of phosphorylation
of IκB in a cell, thereby modulating the activity of
NF-κB in the cell and the expression of a gene regulated
25 by NF-κB.

          The invention also provides methods of
identifying proteins that can interact with an IκB
kinase, including with an IKK subunit, such proteins
which can be a downstream effector of the IKK such as a
30 member of the IκB family of proteins or an upstream
activator or a regulatory subunit of an IKK. Such
proteins that interact with an IKK complex or the IKK
subunit can be isolated, for example, by coprecipitation
with the IKK or by using the IKK subunit as a ligand, and
35 can be involved, for example, in tissue specific

regulation of NF-κB activation and consequent tissue
specific gene expression.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleotide sequence (SEQ ID
5   NO: 1; lower case letter) and deduced amino acid sequence
(SEQ ID NO: 2; upper case letters) of full length human
IKKα subunit of an IKK complex. Nucleotide positions are
indicated to the right and left of the sequence; the "A"
of the ATG encoding the initiator methionine is shown as
10 position 1. Underlined amino acid residues indicate the
peptide portions of the protein ("peptide 1" and
"peptide 2") that were sequenced and used to design
oligonucleotide probes. The asterisk indicates the
sequence encoding the STOP codon.

15   Figure 2 shows a nucleotide sequence (SEQ ID
NO: 14) encoding a full length IKKβ polypeptide (see
Figure 3). Numbers to the left and right of the sequence
indicate nucleotide position number. The initiator ATG
codon is present at nucleotides 36-38 and the first stop
20 codon (TGA) is present at nucleotides 2304-2306.

Figure 3 shows an alignment of the deduced
amino acid sequences of IKKα ("α", SEQ ID NO: 2) and IKKβ
("β", SEQ ID NO: 15). Numbers to the right of the
sequences indicate the respective amino acid positions.
25 Underlined amino acid residues indicate peptide portions
of the IKKβ subunit that were sequenced and used to
search an EST database (see Example III). Vertical bars
between amino acid residues indicate identical amino
acids; two dots between amino acid residues indicates
30 very similar amino acids (e.g., Glu and Asp; Arg and Lys)
and one dot between amino acid residues indicates a
lesser degree of similarity. A dot within an amino acid
sequence indicates a space introduced to maintain

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sequence homology. The kinase domains in the N-terminal
half of the sequences and helix-loop-helix domains in the
C-terminal half of the sequences are bracketed and the
leucine residues involved in the leucine zippers are
5 indicated by the filled circles above the IKKα sequence.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides isolated nucleic
acid molecules encoding polypeptide subunits of human
serine protein kinase complex, the IκB kinase (IKK),
10 which is activated in response to proinflammatory signals
and phosphorylates proteins (IκB's) that bind to and
inhibit the activity of NF-κB transcription factors. For
example, the invention provides an isolated nucleic acid
molecule (SEQ ID NO: 1) encoding a full length human IKKα
15 subunit having the amino acid sequence shown as SEQ ID
NO: 2 (Figure 1). In addition, the invention provides an
isolated nucleic acid molecule (SEQ ID NO: 14; Figure 2)
encoding a full length human IKKβ subunit having the
amino acid sequence shown as SEQ ID NO: 15 (Figure 3).

20 As used herein, the term "isolated," when used
in reference to a nucleic acid molecule of the invention,
means that the nucleic acid molecule is relatively free
from contaminating lipids, proteins, nucleic acids or
other cellular material normally associated with a
25 nucleic acid molecule in a cell. An isolated nucleic
acid molecule of the invention can be obtained, for
example, by chemical synthesis of the nucleotide sequence
shown as SEQ ID NO: 1 or SEQ ID NO: 14 or by cloning the
molecule using methods such as those disclosed in
30 Examples II and III. In general, an isolated nucleic
acid molecule comprises at least about 30% of a sample
containing the nucleic acid molecule, and generally
comprises about 50% or 70% or 90% of a sample, preferably
95% or 98% of the sample. Such an isolated nucleic acid

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molecule can be identified by comparing, for example, a
sample containing the isolated nucleic acid molecule with
the material from which the sample originally was
obtained. Thus, an isolated nucleic acid molecule can be
5   identified, for example, by comparing the relative amount
of the nucleic acid molecule in fraction of a cell lysate
obtained following gel electrophoresis with the relative
amount of the nucleic acid molecule in the cell, itself.

     IKKα and IKKβ have been designated IKK subunits
10 because they are components of an approximately 900 kDa
complex having IκB kinase (IKK) activity and because they
share substantial nucleotide and amino acid sequence
homology. As disclosed herein, IKKα and IKKβ are related
members of a family of IKK catalytic subunits (see
15 Figure 3). The 900 kDa IκB kinase complex can be
isolated in a single step, for example, by
immunoprecipitation using an antibody specific for an IKK
subunit or by using metal ion chelation chromatography
methods (see Example IV). A 300 kDa IKK complex also can
20 be isolated as disclosed herein and has kinase activity
for an IκB substrate (see Example III).

     Nucleic acid molecules related to SEQ ID NO: 1
previously have been described (Connelly and Marcu, Cell.
Mol. Biol. Res. 41:537-549 (1995), which is incorporated
25 herein by reference). For example, Connelly and Marcu
describe a 3466 base pair (bp) nucleic acid molecule
(GenBank Accession #U12473; Locus MMU 12473), which is
incorporated herein by reference), which encodes a full
length mouse polypeptide having an apparent molecular
30 mass of 85 kiloDaltons (kDa) and designated CHUK. A 2146
bp nucleic acid molecule (GenBank Accession #U22512;
Locus HSU 22512), which is incorporated herein by
reference), which encodes a portion of the polypeptide
shown in SEQ ID NO: 2 also was described. However, the
35 amino acid sequence deduced from #U22512 lacks amino

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acids 1 to 31 as shown in SEQ ID NO: 2 and, therefore, is
not a full length protein. In addition, several
nucleotide differences occur in SEQ ID NO: 1 as compared
to the sequence of #U22512, including nucleotide changes
5   that encode different amino acids at positions 543, 604,
679, 680, 684 and 685 of SEQ ID NO: 2; silent nucleotide
changes also occur at codons 665 and 678. The
polypeptides encoded by the nucleotide sequences of
GenBank Accession #U12473 and #U22512 share about 95%
10   identity at the amino acid level and are substantially
similar to that shown in SEQ ID NO: 2. No function has
been demonstrated for the polypeptides described by
Connelly and Marcu, although Regnier et al. (Cell
90:373-383 (1997)) recently have confirmed that human
15   CHUK corresponds to IKKα, as disclosed herein.

A nucleic acid molecule of the invention is
exemplified by the nucleotide sequences shown as SEQ ID
NO: 1, which encodes a full length human IKKα (SEQ ID
NO: 2; Figure 1), the activity of which is stimulated by
20   a cytokine or other proinflammatory signal, and as SEQ ID
NO: 14, which encodes a full length IKKβ (SEQ ID NO: 15).
Due to the degeneracy of the genetic code and in view of
the disclosed amino acid sequence of a full length human
IKKα (SEQ ID NO: 2) and of the IKKβ (SEQ ID NO: 15),
25   additional nucleic acid molecules of the invention would
be well known to those skilled in the art. Such nucleic
acid molecules, respectively, have a nucleotide sequence
that is different from SEQ ID NO: 1 but, nevertheless,
encodes the amino acid sequence shown as SEQ ID NO: 2, or
30   have a nucleotide sequence that is different from SEQ ID
NO: 14 but, nevertheless, encodes the amino acid sequence
shown as SEQ ID NO: 15. Thus, the invention provides a
nucleic acid molecule comprising a nucleotide sequence
encoding the amino acid sequence of a full length human
35   IKKα as shown in SEQ ID NO: 2 or of IKKβ as shown in SEQ
ID NO: 15.

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As used herein, reference to "a nucleic acid
molecule encoding an IKK subunit" indicates 1) the
polynucleotide sequence of one strand of a double
stranded DNA molecule comprising the nucleotide sequence
5   that codes for the IKK subunit and can be transcribed
into an RNA that encodes the IKK subunit, or 2) an RNA
molecule, which can be translated into an IKK subunit.
It is recognized that a double stranded DNA molecule also
comprises a second polynucleotide strand that is
10   complementary to the coding strand and that the
disclosure of a polynucleotide sequence comprising a
coding sequence necessarily discloses the complementary
polynucleotide sequence. Accordingly, the invention
provides polynucleotide sequences, including, for
15   example, polydeoxyribonucleotide or polyribonucleotide
sequences that are complementary to the nucleotide
sequence shown as SEQ ID NO: 1 or as SEQ ID NO: 14, or to
a nucleic acid molecule encoding an IKK catalytic subunit
having the amino acid sequence shown as SEQ ID NO: 2 or
20   as SEQ ID NO: 15, respectively.

As used herein, the term "polynucleotide" is
used in its broadest sense to mean two or more
nucleotides or nucleotide analogs linked by a covalent
bond. The term "oligonucleotide" also is used herein to
25   mean two or more nucleotides or nucleotide analogs linked
by a covalent bond, although those in the art will
recognize that oligonucleotides generally are less than
about fifty nucleotides in length and, therefore, are a
subset within the broader meaning of the term
30   "polynucleotide."

In general, the nucleotides comprising a
polynucleotide are naturally occurring
deoxyribonucleotides, such as adenine, cytosine, guanine
or thymine linked to 2'-deoxyribose, or ribonucleotides
35   such as adenine, cytosine, guanine or uracil linked to

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ribose. However, a polynucleotide also can comprise
nucleotide analogs, including non-naturally occurring
synthetic nucleotides or modified naturally occurring
nucleotides. Such nucleotide analogs are well known in
5   the art and commercially available, as are
polynucleotides containing such nucleotide analogs (Lin
et al., Nucl. Acids Res. 22:5220-5234 (1994); Jellinek et
al., Biochemistry 34:11363-11372 (1995); Pagratis et al.,
Nature Biotechnol. 15:68-73 (1997)). The covalent bond
10   linking the nucleotides of a polynucleotide generally is
a phosphodiester bond. However, the covalent bond also
can be any of numerous other bonds, including a
thiodiester bond, a phosphorothioate bond, a peptide-like
bond or any other bond known to those in the art as
15   useful for linking nucleotides to produce synthetic
polynucleotides (see, for example, Tam et al., Nucl.
Acids Res. 22:977-986 (1994); Ecker and Crooke,
BioTechnology 13:351360 (1995)).

Where it is desired to synthesize a
20   polynucleotide of the invention, the artisan will know
that the selection of particular nucleotides or
nucleotide analogs and the covalent bond used to link the
nucleotides will depend, in part, on the purpose for
which the polynucleotide is prepared. For example, where
25   a polynucleotide will be exposed to an environment
containing substantial nuclease activity, the artisan
will select nucleotide analogs or covalent bonds that are
relatively resistant to the nucleases. A polynucleotide
comprising naturally occurring nucleotides and
30   phosphodiester bonds can be chemically synthesized or can
be produced using recombinant DNA methods, using an
appropriate polynucleotide as a template. In comparison,
a polynucleotide comprising nucleotide analogs or
covalent bonds other than phosphodiester bonds generally
35   will be chemically synthesized, although an enzyme such
as T7 polymerase can incorporate certain types of

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nucleotide analogs and, therefore, can be used to produce
such a polynucleotide recombinantly from an appropriate
template (Jellinek et al., *supra*, 1995).

5   The invention also provides nucleotide
sequences that can specifically hybridize to a nucleic
acid molecule of the invention. Such hybridizing
nucleotide sequences are useful, for example, as probes,
which can hybridize to a nucleic acid molecule encoding
an IKK catalytic subunit and allow the identification of
10   the nucleic acid molecule in a sample. A nucleotide
sequence of the invention is characterized, in part, in
that it is at least nine nucleotides in length, such
sequences being particularly useful as primers for the
polymerase chain reaction (PCR), and can be at least
15   fourteen nucleotides in length or, if desired, at least
seventeen nucleotides in length, such nucleotide
sequences being particularly useful as hybridization
probes, although such sequences also can be used for PCR.
A nucleotide sequence of the invention can comprise at
20   least six nucleotides 5' to nucleotide position 92 as
shown in SEQ ID NO: 1 (Figure 1), preferably at least
nine nucleotides 5' to position 92, or more as desired,
where SEQ ID NO: 1 is shown in the conventional manner
from the 5'-terminus (Figure 1; upper left) to the
25   3'-terminus. Such nucleotide sequences of the invention
are particularly useful in methods of diagnosing a
pathology, for example, a human disease, characterized by
aberrant IKK activity. For convenience, such nucleotide
sequences can comprise a kit, which can be made
30   commercially available and can provide a standardized
diagnostic assay.

        A nucleic acid molecule encoding an IKKα such
as the nucleotide sequence shown in SEQ ID NO: 1 diverges
from the sequence encoding the mouse homolog (GenBank
35   Accession #U12473) in the region encoding amino acid 30.

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Thus, a nucleotide sequence comprising nucleotides 88 to
90 as shown in SEQ ID NO: 1, which encodes amino acid 30
of human IKKα, can be particularly useful, for example,
for identifying the presence of a nucleic acid molecule
5   encoding a human IKKα in a sample. Furthermore, based on
a comparison of SEQ ID NO: 1 with SEQ ID NO: 14, the
skilled artisan readily can select nucleotide sequences
that can hybridize with a nucleic acid molecule encoding
a human IKKα or a human IKKβ or both by designing the
10 sequence to contain conserved or non-conserved nucleotide
sequences, as desired. For example, selection of a
nucleotide sequence that is highly conserved among SEQ ID
NO: 1 and SEQ ID NO: 14 can allow the identification of
related members of the IKK subunit family of proteins.
15 In comparison, selection of a nucleotide sequence that is
present, for example, in SEQ ID NO: 14, but that is not
present in SEQ ID NO: 1 or that shares only minimal
homology can allow identification of the expression of
SEQ ID NO: 14 in a cell, irrespective of whether SEQ ID
20 NO: 1 also is expressed in the cell. It should be
recognized, however, that a nucleotide sequence of the
invention readily is identifiable in comparison to
GenBank Accession #U12473 or #U22512 in that a nucleotide
sequence of the invention is not the nucleotide sequence
25 of GenBank Accession #U12473 or #U22512.

A nucleotide sequence of the invention can
comprise a portion of a coding sequence of a nucleic acid
molecule encoding an IKK subunit or of a sequence
complementary thereto, depending on the purpose for which
30 the nucleotide sequence is to be used. In addition, a
mixture of a coding sequence and its complementary
sequence can be prepared and, if desired, can be allowed
to anneal to produce double stranded molecules.

The invention also provides antisense nucleic
35 acid molecules, which are complementary to a nucleic acid

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molecule encoding an IKK subunit and can bind to and
inhibit the expression of the nucleic acid molecule. As
disclosed herein, expression of an antisense molecule
complementary to the nucleotide sequence shown in SEQ ID
5   NO: 1 inhibited the cytokine inducible expression of an
NF-κB dependent reporter gene in a cell (Example II.B.).
Thus, an antisense molecule of the invention can be
useful for decreasing IKK activity in a cell, thereby
reducing or inhibiting the level of NF-κB mediated gene
10   expression. These experiments were performed twenty-four
hours after the cells were transfected (Example II.B.).
Expression of the antisense molecule in the cell also
resulted in a decreased level of IKKα activity as
compared to vector transfected control cells, indicating
15   that the IKKα has a relatively short half life.
Antisense nucleic acid molecules specific for IKKα or for
IKKβ or for both can be designed based on the criteria
discussed above for the selection of hybridizing
nucleotide sequences.

20   An antisense nucleic acid molecule of the
invention can comprise a sequence complementary to the
entire coding sequence of an IKK catalytic subunit such
as a sequence complementary to SEQ ID NO: 1 or SEQ ID NO:
14, provided the antisense sequence is not complementary
25   in its entirety to the sequences of GenBank Accession
#U12473 or #U22512. In addition, a nucleotide sequence
complementary to a portion of a nucleic acid molecule
encoding an IKK subunit can be useful as an antisense
molecule, particularly a nucleotide sequence
30   complementary to nucleotides -35 to 92 of SEQ ID NO: 1
or, for example, a nucleotide sequence comprising at
least 9 nucleotides on each side of the ATG encoding the
initiator methionine (complementary to positions -9 to 12
of SEQ ID NO: 1) or, if desired, at least 17 nucleotides
35   on each side of the ATG codon (complementary to positions

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-17 to 20 of SEQ ID NO: 1), or to the corresponding
sequences of SEQ ID NO: 14.

Antisense methods involve introducing the
nucleic acid molecule, which is complementary to and can
5   hybridize to the target nucleic acid molecule, into a
cell. An antisense nucleic acid molecule can be a
chemically synthesized polynucleotide, which can be
introduced into the target cells by methods of
transfection, or can be expressed from a plasmid or viral
10   vector, which can be introduced into the cell and stably
or transiently expressed using well known methods (see,
for example, Sambrook et al., Molecular Cloning: A
laboratory manual (Cold Spring Harbor Laboratory Press
1989); Ausubel et al., Current Protocols in Molecular
15   Biology (Green Publ., NY 1989), each of which is
incorporated herein by reference). One in the art would
know that the ability of an antisense (or other
hybridizing) nucleotide sequence to specifically
hybridize to the target nucleic acid sequence depends,
20   for example, on the degree of complementarity shared
between the sequences, the GC content of the hybridizing
molecules, and the length of the antisense nucleic acid
sequence, which can be at least ten nucleotides in
length, generally at least thirty nucleotides in length
25   or at least fifty nucleotides in length, and can be up to
the full length of a nucleotide sequence of SEQ ID NO: 1
or SEQ ID NO: 14 or a nucleotide sequence encoding an IKK
subunit as shown in SEQ ID NO: 2 or in SEQ ID NO: 15 (see
Sambrook et al., supra, 1989).

30   The invention also provides vectors comprising
a nucleic acid molecule of the invention and host cells,
which are appropriate for maintaining such vectors.
Vectors, which can be cloning vectors or expression
vectors, are well known in the art and commercially
35   available. An expression vector comprising a nucleic

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acid molecule of the invention, which can encode an IKK-α
or can be an antisense molecule, can be used to express
the nucleic acid molecule in a cell.

5   In general, an expression vector contains the
expression elements necessary to achieve, for example,
sustained transcription of the nucleic acid molecule,
although such elements also can be inherent to the
nucleic acid molecule cloned into the vector. In
particular, an expression vector contains or encodes a
10   promoter sequence, which can provide constitutive or, if
desired, inducible expression of a cloned nucleic acid
sequence, a poly-A recognition sequence, and a ribosome
recognition site, and can contain other regulatory
elements such as an enhancer, which can be tissue
15   specific. The vector also contains elements required for
replication in a procaryotic or eukaryotic host system or
both, as desired. Such vectors, which include plasmid
vectors and viral vectors such as bacteriophage,
baculovirus, retrovirus, lentivirus, adenovirus, vaccinia
20   virus, semliki forest virus and adeno-associated virus
vectors, are well known and can be purchased from a
commercial source (Promega, Madison WI; Stratagene, La
Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be
constructed by one skilled in the art (see, for example,
25   Meth. Enzymol., Vol. 185, D.V. Goeddel, ed. (Academic
Press, Inc., 1990); Jolly, Canc. Gene Ther. 1:51-64
(1994); Flotte, J. Bioenerg. Biomemb. 25:37-42 (1993);
Kirshenbaum et al., J. Clin. Invest 92:381-387 (1993),
which is incorporated herein by reference).

30   A nucleic acid molecule, including a vector,
can be introduced into a cell by any of a variety of
methods known in the art (Sambrook et al., *supra*, 1989,
and in Ausubel et al., Current Protocols in Molecular
Biology, John Wiley and Sons, Baltimore, MD (1994), which
35   is incorporated herein by reference). Such methods

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include, for example, transfection, lipofection,
microinjection, electroporation and infection with
recombinant vectors or the use of liposomes.

 5   Introduction of a nucleic acid molecule by
infection with a viral vector is particularly
advantageous in that it can efficiently introduce the
nucleic acid molecule into a cell *ex vivo* or *in vivo*.
Moreover, viruses are very specialized and typically
infect and propagate in specific cell types. Thus, their
10 natural specificity can be used to target the nucleic
acid molecule contained in the vector to specific cell
types. For example, a vector based on HIV-1 can be used
to target an antisense IKK subunit molecule to HIV-1
infected cells, thereby reducing the phosphorylation of
15 IκB, which can decrease the high level of constitutive
NF-κB activity present in HIV-1 infected cells. Viral or
non-viral vectors also can be modified with specific
receptors or ligands to alter target specificity through
receptor mediated events.

20   A nucleic acid molecule also can be introduced
into a cell using methods that do not require the initial
introduction of the nucleic acid molecule into a vector.
For example, a nucleic acid molecule encoding an IKK
catalytic subunit can be introduced into a cell using a
25 cationic liposomes, which also can be modified with
specific receptors or ligands as described above
(Morishita et al., J. Clin. Invest., 91:2580-2585 (1993),
which is incorporated herein by reference; see, also,
Nabel et al., *supra*, 1993)). In addition, a nucleic acid
30 molecule can be introduced into a cell using, for
example, adenovirus-polylysine DNA complexes (see, for
example, Michael et al., J. Biol. Chem., 268:6866-6869
(1993), which is incorporated herein by reference).
Other methods of introducing a nucleic acid molecule into
35 a cell such that the encoded IKK subunit or antisense

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nucleic acid molecule can be expressed are well known
(see, for example, Goeddel, *supra*, 1990).

Selectable marker genes encoding, for example,
a polypeptide conferring neomycin resistance (Neo^R) also
5   are readily available and, when linked to a nucleic acid
molecule of the invention or incorporated into a vector
containing the nucleic acid molecule, allows for the
selection of cells that have incorporated the nucleic
acid molecule. Other selectable markers such as that
10 conferring hygromycin, puromycin or ZEOCIN (Invitrogen)
resistance are known to those in the art of gene transfer
can be used to identify cells containing the nucleic acid
molecule, including the selectable marker gene.

A "suicide" gene also can be incorporated into
15 a vector so as to allow for selective inducible killing
of a cell containing the gene. A gene such as the herpes
simplex virus thymidine kinase gene (TK) can be used as a
suicide gene to provide for inducible destruction of such
cells. For example, where it is desired to terminate the
20 expression of an introduced nucleic acid molecule
encoding IKK or an antisense IKK subunit molecule in
cells containing the nucleic acid molecule, the cells can
be exposed to a drug such as acyclovir or gancyclovir,
which can be administered to an individual.

25   Numerous methods are available for transferring
nucleic acid molecules into cultured cells, including the
methods described above. In addition, a useful method
can be similar to that employed in previous human gene
transfer studies, where tumor infiltrating lymphocytes
30 (TILs) were modified by retroviral gene transduction and
administered to cancer patients (Rosenberg et al., New
Engl. J. Med. 323:570-578 (1990)). In that Phase I
safety study of retroviral mediated gene transfer, TILs
were genetically modified to express the Neomycin

resistance (Neo^R) gene. Following intravenous infusion,
polymerase chain reaction analyses consistently found
genetically modified cells in the circulation for as long
as two months after administration. No infectious

5   retroviruses were identified in these patients and no
side effects due to gene transfer were noted in any
patients. These retroviral vectors have been altered to
prevent viral replication by the deletion of viral gag,
pol and env genes. Such a method can also be used *ex*

10 *vivo* to transduce cells taken from a subject (see
Anderson et al., U.S. Patent No. 5,399,346, issued
March 21, 1995, which is incorporated herein by
reference).

        When retroviruses are used for gene transfer,
15 replication competent retroviruses theoretically can
develop due to recombination of retroviral vector and
viral gene sequences in the packaging cell line utilized
to produce the retroviral vector. Packaging cell lines
in which the production of replication competent virus by
20 recombination has been reduced or eliminated can be used
to minimize the likelihood that a replication competent
retrovirus will be produced. Hence, all retroviral
vector supernatants used to infect cells will be screened
for replication competent virus by standard assays such
25 as PCR and reverse transcriptase assays.

        To function properly, a cell requires the
precise regulation of expression of nearly all genes.
Such gene regulation is accomplished by activation or
repression of transcription by various transcription
30 factors, which interact directly with regulatory
sequences on nuclear DNA. The ability of transcription
factors to bind DNA or activate or repress transcription
is regulated in response to external stimuli. In the
case of the transcription factor NF-κB, critical factors
35 involved in the signaling pathway mediating its

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activation have not been identified (Verma, et al., <u>Genes Devel.</u> 9:2723-2735 (1995); Baeuerle and Baltimore, <u>Cell</u> 87:13-20 (1996)).

5   NF-κB is a member of the Rel family of transcription factors, which are present in most if not all animal cells (Thanos and Maniatis, <u>Cell</u> 80:629-532 (1995)). Rel proteins, which include, for example, RelA (p65), c-Rel, p50, p52 and the *Drosophila* dorsal and Dif gene products, are characterized by region of about 300
10 amino acids sharing approximately 35% to 61% homology ("Rel homology domain"). The Rel homology domain includes DNA binding and dimerization domains and a nuclear localization signal. Rel proteins are grouped into one of two classes, depending on whether the protein
15 also contains a transcriptional activation domain (Siebenlist et al., <u>Ann. Rev. Cell Biol</u>. 10:405-455 (1994)).

Rel proteins can from homodimers or heterodimers, which can be transcriptionally activating
20 depending on the presence of a transactivation domain. The most common Rel/NF-κB dimer, which is designated "NF-κB," is a p50/p65 heterodimer that can activate transcription of genes containing the appropriate κB binding sites. p50/p65 NF-κB is present in most cell
25 types and is considered the prototype of the Rel/NF-κB family of transcription factors. Different dimers vary in their binding to different κB elements, kinetics of nuclear translocation and levels of expression in a tissue (Siebenlist et al., *supra*, 1994). As used herein,
30 the term "Rel/NF-κB" is used to refer generally to the Rel family of transcription factors, and the term "NF-κB" is used to refer specifically to the Rel/NF-κB factor consisting of a p50/p65 heterodimer.

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NF-κB originally was identified by its ability
to bind a specific DNA sequence present in the
immunoglobulin κ light chain gene enhancer, the "κB
element" (Sen and Baltimore, Cell 46:705-709 (1986)).
5   The κB element has been identified in numerous cellular
and viral promotors, including promotors present in human
immunodeficiency virus-1 (HIV-1); immunoglobulin
superfamily genes such as the MHC class 1 (H-2κ) gene;
cytokine genes such as the tumor necrosis factor α
10 (TNFα), interleukin-1β (IL-1β), IL-2, IL-6 and the
granulocyte-macrophage colony stimulating factor (GM-CSF)
gene; chemokine genes such as RANTES and IL-8; and cell
adhesion protein genes such as E-selectin. The κB
element exhibits dyad symmetry and each half site of the
15 element likely is bound by one subunit of an NF-κB dimer.

In the absence of an appropriate signaling
stimulus, a Rel/NF-κB is maintained in the cytoplasm in
an inactive form complexed with an IκB protein.
Rel/NF-κB transcriptional activity is induced by numerous
20 pathogenic events or stresses, including cytokines,
chemokines, viruses and viral products, double stranded
RNA, bacteria and bacterial products such as
lipopolysaccharide (LPS) and toxic shock syndrome
toxin-1, mitogens such as phorbol esters, physical and
25 oxidative stresses, and chemical agents such as okadaic
acid and cycloheximide (Thanos and Maniatis, *supra*, 1995;
Siebenlist et al., *supra*, 1994). Significantly, the
expression of genes encoding agents such as TNFα, IL-1,
IL-6, interferon-β and various chemokines, which induce
30 NF-κB activity, are, themselves, induced by NF-κB,
resulting in amplification of their signal by a positive,
self-regulatory loop (Siebenlist et al., *supra*, 1994).
Phorbol esters, which activate T cells, also activate
NF-κB and immunosuppressants such as cyclosporin A
35 inhibit activation of T cells through T cell receptor

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mediated signals (Baldwin, Ann. Rev. Immunol. 14:649-681 (1996), which is incorporated herein by reference).

Regulation of specific genes by NF-κB can require interaction of NF-κB with one or more other DNA
5 binding proteins. For example, expression of E-selectin requires an interaction of NF-κB, the bZIP protein ATF-2 and HMG-I(Y), and expression of the IL-2 receptor α gene requires an interaction of NF-κB, HMG-I(Y) and the ets-like protein, ELF-1 (Baldwin, supra, 1996).

10 The numerous agents that induce activation of NF-κB likely act through various converging signal transduction pathways, including pathways involving activation of protein kinase C, Raf kinase and tyrosine kinases. The ability of antioxidants to inhibit NF-κB
15 activation by various inducing agents suggests that reactive oxygen species are a converging point of such pathways (Siebenlist et al., supra, 1994).

Upon activation by an appropriate inducing agent, a Rel/NF-κB dimer is translocated into the
20 nucleus, where it can activate gene transcription. The subcellular localization of a Rel/NF-κB is controlled by specific inhibitory proteins ("inhibitors of Rel/NF-κB" or "IκB's"), which noncovalently bind the Rel/NF-κB and mask its nuclear localization signal (NLS), thereby
25 preventing nuclear uptake. Various IκB's, including, for example, IκBα, IκBβ, Bcl-3 and the Drosophila cactus gene product, have been identified (Baeuerle and Baltimore, supra, 1996). In addition, Rel precursor proteins, such as p105 and p100, which are precursors of p50 and p52,
30 respectively, function as IκB's (Siebenlist et al., supra, 1994). IκBα and IκBβ are expressed in most cell types and generally bind p65- and c-Rel-containing Rel/NF-κB dimers. Other IκB's appear to be expressed in

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a tissue specific manner (Thompson et al., <u>Cell</u> 80:573-582 (1995)).

IκB proteins are characterized by the presence of 5 to 8 ankyrin repeat domains, each about 30 amino
5   acids, and a C-terminal PEST domain. For example, IκBα contains a 70 amino acid N-terminal domain, a 205 amino acid internal domain containing the ankyrin repeats, and a 42 amino acid C-terminal domain containing the PEST domain (Baldwin, *supra*, 1996). Although IκB proteins
10 interact through their ankyrin repeats with the Rel homology domain of Rel/NF-κB dimers, binding of particular IκB proteins with particular Rel/NF-κB proteins appears to be relatively specific. For example, IκBα and IκBβ associate primarily with RelA- and c-Rel-
15 containing Rel/NF-κB dimers, thereby blocking their nuclear localization signal. The binding of an IκB to NF-κB also interferes with the ability of NF-κB to bind DNA. However, whereas IκBα is phosphorylated following exposure of cells to tumor necrosis factor (TNF), IL-1,
20 bacterial lipopolysaccharide (LPS) or phorbol esters, IκBβ is phosphorylated in certain cell types only in response to LPS or IL-1 (Baldwin, *supra*, 1996). However, in other cell types, IκBβ is phosphorylated in response to the same signals that induce IκBα, although with
25 slower kinetics than IκBα (DiDonato et al., <u>Mol. Cell.</u> <u>Biol.</u> 16:1295-1304 (1996), which is incorporated herein by reference).

Formation of a complex between an IκB protein and a Rel protein is due to an interaction of the ankyrin
30 domains with a Rel homology domain (Baeuerle and Baltimore, *supra*, 1996). Upon exposure to an appropriate stimulus, the IκB portion of the complex is rapidly degraded and the Rel/NF-κB portion becomes free to translocate to the cell nucleus. Thus, activation of a
35 Rel/NF-κB does not require *de novo* protein synthesis and,

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therefore, occurs extremely rapidly. Consequently,
activation of gene expression due to a Rel/NF-κB can be
exceptionally rapid and provides an effective means to
respond to an external stimulus. Such a rapid response
5   of Rel/NF-κB transcription factors is particularly
important since these factors are involved in the
regulation of genes involved in the immune, inflammatory
and acute phase responses, including responses to viral
and bacterial infections and to various stresses.

10   Upon exposure of a cell to an appropriate
inducing agent, IκBα, for example, is phosphorylated at
serine residue 32 (Ser-32) and Ser-36 (Haskill et al.,
Cell 65:1281-1289 (1991)). Phosphorylation of IκBα
triggers its rapid ubiquitination, which results in
15   proteasome-mediated degradation of the inhibitor and
translocation of active NF-κB to the nucleus (Brown et
al., Science 267:1485-1488 (1995); Scherer et al., Proc.
Natl. Acad. Sci., USA. 92:11259-11263 (1995); DiDonato et
al., supra, 1996; DiDonato et al., Mol. Cell. Biol.
20   15:1302-1311 (1995); Baldi et al., J. Biol. Chem.
271:376-379 (1996)). The same mechanism also accounts
for IκBβ degradation (DiDonato et al., supra, 1996).

         Rel/NF-κB activation can be transient or
persistent, depending on the inducing agent and the IκB
25   that is phosphorylated. For example, exposure of a cell
to particular cytokines induces IκBα phosphorylation and
degradation, resulting in NF-κB activation, which induces
the expression of various genes, including the gene
encoding IκBα. The newly expressed IκBα then binds to
30   NF-κB in the nucleus, resulting in its export to the
cytoplasm and inactivation and, therefore, a transient
NF-κB mediated response. In comparison, bacterial LPS
induces IκBβ phosphorylation, resulting in NF-κB
activation. However, the IκBβ gene is not induced by

NF-κB and, as a result, activation of NF-κB is more persistent (Thompson et al., *supra*, 1995).

A constitutively active multisubunit kinase of approximately 700 kDa phosphorylates IκBα at Ser-32 and

5 Ser-36 and, in some cases, requires polyubiquitination for activity (Chen et al., <u>Cell</u> 84:853-862 (1996); Lee et al., <u>Cell</u> 88:213-222 (1997)). The mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1) phosphorylates several proteins that copurify with this complex and have

10 molecular weights of approximately 105 kDa, 64 kDa and 54 kDa; three other copurifying proteins having molecular weights of about 200 kDa, 180 kDa and 120 kDa are phosphorylated in the absence of MEKK1 (Lee et al., *supra*, 1997). However, a catalytically inactive MEKK1

15 mutant, which can block TNFα mediated activation of the jun kinase, does not block NF-κB activation (Liu et al., <u>Cell</u> 87:565-576 (1996)).

Overexpression of MEKK1 also induces the site-specific phosphorylation of IκBα *in vivo* and can directly

20 activate IκBα *in vitro* by an ubiquitin-independent mechanism. However, MEKK1 did not phosphorylate IκBα at Ser-32 and Ser-36 in the *in vitro* experiments, indicating that it is not an IκBα kinase, but may act upstream of IκBα kinase in a signal transduction pathway (Lee et al.,

25 *supra*, 1997).

In addition to the above described ubiquitin dependent kinase 700 kDa complex, an ubiquitin independent 700 kDa complex, as well as an ubiquitin independent 300 kDa kinase complex phosphorylates IκBα

30 Ser-32 and Ser-36, but not a mutant containing threonines substituted for these serines (Baeuerle and Baltimore, *supra*, 1996). The specific polypeptides responsible for the IκB kinase activity of these complexes have not been described.

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A double stranded RNA-dependent protein kinase (PKR) that phosphorylates IκBα *in vitro* has been described (Kumar et al., Proc. Natl. Acad. Sci., *USA* 91:6288-6292 (1994)). Moreover, an antisense PKR DNA

5   molecule prevented NF-κB activation by double stranded RNA, but did not prevent NF-κB activation by TNFα (Maran et al., Science 265:789-792 (1995)). Casein kinase II (CKII) also can interact with and phosphorylate IκBα, although weakly as compared to CKII phosphorylation of

10 casein, and the Ser-32 and Ser-36 residues in IκBα represent CKII phosphorylation sites (Roulston et al., *supra*, 1995). However, all of the inducers of NF-κB activity do not stimulate these protein kinases to phosphorylate IκB, indicating that, if they are involved

15 in NF-κB activation, these kinases, like MEKK1, operate upstream of the IκB kinase. Thus, a rapidly stimulated IκB kinase that directly phosphorylates IκBα on Ser-32 and Ser-36 and results in activation of NF-κB has not been identified.

20   A putative serine-threonine protein kinase has been identified in mouse cells by probing for nucleic acid molecules that encode proteins containing a consensus helix-loop-helix domain, which is involved in protein-protein interactions (Connelly and Marcu, *supra*,

25 1995). This putative kinase, which is ubiquitously expressed in various established cell lines, but differentially expressed in normal mouse tissues, was named CHUK (conserved helix-loop-helix ubiquitous kinase; GenBank Accession #U12473). In addition, a nucleic acid

30 molecule (GenBank Accession #U22512) encoding a portion of a human CHUK protein that is 93% identical at the nucleotide level (95% identical at the amino acid level) with the mouse CHUK also was identified. However, neither the function of a CHUK protein in a cell nor a

35 potential substrate for the putative kinase was described.

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　　　　The present invention provides an isolated IκB
kinase (IKK), including isolated full length IKK
catalytic subunits. For example, the invention provides
an isolated 300 kDa or 900 kDa complex, which comprises
5   an IKKα or an IKKβ subunit and has IκB kinase activity
(see Examples I, III and IV). In addition, the invention
provides is an isolated human IKKα catalytic subunit (SEQ
ID NO: 2; Example II), which contains a previously
undescribed N-terminal amino acid sequence and
10   essentially the C-terminal region of human CHUK (Connelly
and Marcu, *supra*, 1995) and phosphorylates IκBα on Ser-32
and Ser-36 and IκBβ on Ser-19 and Ser-23 (DiDonato et
al., *supra*, 1996; see, also, Regnier et al., *supra*,
1997). The invention also provides an isolated IKKβ
15   catalytic subunit (SEQ ID NO: 15; Example III), which
shares greater than 50% amino acid sequence identity with
IKKα, including conserved homology in the kinase domain,
helix-loop-helix domain and leucine zipper domain.

　　　　As used herein, the term "isolated," when used
20   in reference to an IκB kinase complex or to an IKK
catalytic subunit of the invention, means that the
complex or the subunit is relatively free from
contaminating lipids, proteins, nucleic acids or other
cellular material normally associated with an IKK in a
25   cell. An isolated 900 kDa IκB kinase complex or 300 kDa
complex can be isolated, for example, by
immunoprecipitation using an antibody that binds to an
IKK catalytic subunit (see Examples III and IV). In
addition, an isolated IKK subunit can be obtained, for
30   example, by expression of a recombinant nucleic acid
molecule such as SEQ ID NO: 1 or SEQ ID NO: 14, or can be
isolated from a cell by a method comprising affinity
chromatography using ATP or IκB as ligands (Example I) or
using an anti-IKK subunit antibody. An isolated IKK
35   complex or IKK subunit comprises at least 30% of the
material in a sample, generally about 50% or 70% or 90%

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of a sample, and preferably about 95% or 98% of a sample, as described above with respect to nucleic acids.

The amino acid sequences for MEKK1 (GenBank Accession # U48596; locus RNU48596), PKR (GenBank
5 Accession # M35663; locus HUMP68A) and CKII (GenBank Accession # M55268 J02924; locus HUMA1CKII) are different from the sequences of the IKK subunits disclosed herein (SEQ ID NO: 2 and SEQ ID NO: 15) and, therefore, are distinguishable from the present invention. In addition,
10 a full length human IKKα of the invention is distinguishable from the partial human CHUK polypeptide sequence in that the partial human CHUK polypeptide (Connelly and Marcu, *supra*, 1995; GenBank Accession #22512) lacks amino acids 1 to 31 as shown in SEQ ID
15 NO: 2. As disclosed herein, a polypeptide having the amino acid sequence of the partial human CHUK polypeptide does not have IκB kinase activity when expressed in a cell, indicating that some or all of amino acid residues 1 to 31 are essential for kinase activity.

20   A full length IKK catalytic subunit of the invention is exemplified by human IKKα, which has an apparent molecular mass of about 85 kDa and phosphorylates IκBα on Ser-32 and Ser-36. An IKK catalytic subunit of the invention also is exemplified by
25 IKKβ, which is an 87 kDa polypeptide that shares substantial amino acid sequence homology with IKKα (Figure 3). As used herein, the term "full length," when used in reference to an IKK subunit of the invention, means a polypeptide having an amino acid sequence of an
30 IKK subunit expressed normally in a cell. Such a normally expressed IKK polypeptide begins with a methionine residue at its N-terminus (Met-1; Figure 3), the Met-1 being encoded by the initiator ATG (AUG) codon, and ends as a result of the termination of translation
35 due to the presence of a STOP codon. A full length human

IKK catalytic subunit can be a native IKK polypeptide, which is isolated from a cell, or can be produced using recombinant DNA methods such as by expressing the nucleic acid molecule shown as SEQ ID NO: 1 or SEQ ID NO: 14.

5   The apparent molecular mass of an isolated IKK subunit can be measured using routine methods such as polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate (SDS-PAGE) or column chromatography performed under reducing and denaturing
10 conditions. In addition, the ability of an IKK subunit to phosphorylate IκBα on Ser-32 and Ser-36 can be identified using the methods disclosed herein.

          With regard to the disclosed 85 kDa and 87 kDa apparent molecular masses of human IKKα and IKKβ, it is
15 recognized that the apparent molecular mass of a previously unknown protein as determined, for example, by SDS-PAGE is an estimate based on the relative migration of the unknown protein as compared to the migration of several other proteins having known molecular masses.
20 Thus, one investigator reasonably can estimate, for example, that an unknown protein has an apparent molecular mass of 82 kDa, whereas a second investigator, looking at the same unknown protein under substantially similar conditions, reasonably can estimate that the
25 protein has an apparent molecular mass of 87 kDa. Accordingly, reference herein to an IκB kinase having an apparent molecular mass of "about 85 kDa" indicates that the kinase migrates by SDS-PAGE in an 8% gel under reducing conditions in the range of 80 kDa to 90 kDa,
30 preferably in the range of 82 kDa to 87 kDa. Furthermore, reference herein to an 87 kDa IKKβ indicates that IKKβ has a relatively higher apparent molecular mass than the 85 kDa apparent molecular mass of IKKα.

An IKK catalytic subunit of the invention is
exemplified by the isolated full length polypeptide
comprising the amino acid sequence shown as SEQ ID NO: 2
or SEQ ID NO: 15. In addition, the invention provides
5   peptide portions of an IKK subunit polypeptide, wherein
such peptide portions contain at least three contiguous
amino acids as shown in SEQ ID NO: 2 or SEQ ID NO: 15,
and generally contain at least six contiguous amino acids
or, if desired, at least nine contiguous amino acids, as
10   provided herein. Thus, the invention provides peptide
portions of IKKα, containing, for example, at least three
contiguous amino acids of SEQ ID NO: 2, including amino
acid residue 30, preferably at least four contiguous
amino acids, including amino acid residue 30, and more
15   preferably at least six contiguous amino acids, including
amino acid residue 30. The invention also provides a
peptide portion of IKKβ comprising at least three
contiguous amino acids, generally six contiguous amino
acids, and preferably ten contiguous amino acids of SEQ
20   ID NO: 15. It is recognized, however, that a peptide of
the invention does not consist of a polypeptide disclosed
as GenBank Accession #U12473 or #U22512.

A peptide portion of an IKK subunit generally
is a tripeptide or larger, preferably a hexapeptide or
25   larger, and more preferably a decapeptide or larger, up
to a contiguous amino acid sequence having a maximum
length that lacks one or more N-terminal or C-terminal
amino acids of the full length polypeptide (SEQ ID NO: 2
or SEQ ID NO: 15). Thus, a peptide portion of IKKα
30   having the amino acid sequence shown as SEQ ID NO: 2 can
be from three amino acids long to 744 amino acids long,
which is one residue less than the full length
polypeptide, except as provided above.

A peptide portion of an IKK subunit polypeptide
35   of the invention can be produced by any of several

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methods well known in the art. For example, a peptide
portion of an IKK subunit can be produced by enzymatic
cleavage of an IKK subunit protein, which has been
isolated from a cell, using a proteolytic enzyme such as
5   trypsin, chymotrypsin, Lys-C or the like, or combinations
of such enzymes. Such proteolytic cleavage products can
be isolated using methods as disclosed in Example I, to
obtain peptide portions of IKKα and IKKβ, for example. A
peptide portion of an IKK subunit also can be produced
10   using methods of solution or solid phase peptide
synthesis or can be expressed from a nucleic acid
molecule such as a portion of the coding region of the
nucleic acid sequence shown as SEQ ID NO: 1 or SEQ ID
NO: 14, or can be purchased from a commercial source.

15   A peptide portion of an IKK subunit can
comprise the kinase domain of the IKK subunit and,
therefore, can have the ability to phosphorylate an IκB
protein. For example, a peptide portion of SEQ ID NO: 2
comprising amino acids 15 to 301 has the characteristics
20   of a serine-threonine protein kinase domain (Hanks and
Quinn, Meth. Enzymol. 200:38-62 (1991), which is
incorporated herein by reference). Such a peptide
portion of an IKK subunit can be examined for kinase
activity by determining that it can phosphorylate IκBα at
25   Ser-32 and Ser-36 or IκBβ at Ser-19 and Ser-23, using
methods as disclosed herein. In addition, a peptide
portion of an IKK subunit can comprise an immunogenic
amino acid sequence of the polypeptide and, therefore,
can be useful for eliciting production of an antibody
30   that can specifically bind the IKK subunit or to an IKK
complex comprising the subunit, particularly to an
epitope comprising amino acid residue 30 as shown in SEQ
ID NO: 2 or to an epitope of SEQ ID NO: 15, provided said
epitope is not present in a CHUK protein. Accordingly,
35   the invention also provides anti-IKK antibodies, which
specifically bind to an epitope of an IKK complex,

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particularly an IKK catalytic subunit, and to IKK subunit
binding fragments of such antibodies. In addition, the
invention provides cell lines producing anti-IKK
antibodies or IKK-binding fragments of such antibodies.

5   As used herein, the term "antibody" is used in
its broadest sense to include polyclonal and monoclonal
antibodies, as well as antigen binding fragments of such
antibodies. With regard to an anti-IKK antibody of the
invention, the term "antigen" means an IKK catalytic
10 subunit protein, polypeptide or peptide portion thereof,
or an IKK complex comprising an IKK catalytic subunit
protein, polypeptide or peptide portion thereof. Thus,
it should be recognized that, while an anti-IKK antibody
can bind to and, for example, immunoprecipitate an IKK
15 complex, the antibody specifically binds an epitope
comprising at least a portion of an IKK catalytic
subunit. An antibody of the invention also can be used
to immunoprecipitate an IKK subunit, free of the IKK
complex.

20   An anti-IKK antibody, or antigen binding
fragment of such an antibody, is characterized by having
specific binding activity for an epitope of an IKK
subunit of at least about 1×10^5 M^{-1}, generally, at least
about 1×10^6 M^{-1}. Thus, Fab, $F(ab')_2$, Fd and Fv fragments
25 of an anti-IKK antibody, which retain specific binding
activity for an IKK subunit, are included within the
definition of an antibody. In particular, an anti-IKK
antibody can react with an epitope comprising the
N-terminus of IKKα or with an epitope of IKKβ, but not to
30 a polypeptide having an amino acid sequence shown as
residues 32 to 745 of SEQ ID NO: 2.

            The term "antibody" as used herein includes
naturally occurring antibodies as well as non-naturally
occurring antibodies, including, for example, single

chain antibodies, chimeric, bifunctional and humanized
antibodies, as well as antigen-binding fragments thereof.
Such non-naturally occurring antibodies can be
constructed using solid phase peptide synthesis, can be
5   produced recombinantly or can be obtained, for example,
by screening combinatorial libraries consisting of
variable heavy chains and variable light chains as
described by Huse et al., Science 246:1275-1281 (1989),
which is incorporated herein by reference. These and
10 other methods of making, for example, chimeric,
humanized, CDR-grafted, single chain, and bifunctional
antibodies are well known to those skilled in the art
(Winter and Harris, Immunol. Today 14:243-246 (1993);
Ward et al., Nature 341:544-546 (1989) ; Harlow and Lane,
15 Antibodies: A laboratory manual (Cold Spring Harbor
Laboratory Press, 1988); Hilyard et al., Protein
Engineering: A practical approach (IRL Press 1992);
Borrabeck, Antibody Engineering, 2d ed. (Oxford
University Press 1995); each of which is incorporated
20 herein by reference).

An anti-IKK antibody of the invention can be
raised using an isolated IKK subunit or a peptide portion
thereof and can bind to a free, uncomplexed form of IKK
subunit or can bind to IKK subunit when it is associated
25 with a 300 kDa or 900 kDa IKK complex. In addition, an
anti-IKK antibody of the invention can be raised against
an isolated 300 kDa or 900 kDa IκB kinase complex, which
can be obtained as disclosed herein. For convenience, an
antibody of the invention is referred to generally herein
30 as an "anti-IκB kinase antibody" or an "anti-IKK
antibody." However, the skilled recognize that the
various antibodies of the invention will have unique
antigenic specificities, for example, for a free or
complexed IKK subunit, or both, or for a 300 kDa or
35 900 kDa IκB kinase complex, or both.

Anti-IKK antibodies can be raised using as an immunogen an isolated full length IKK catalytic subunit, which can be prepared from natural sources or produced recombinantly, or a peptide portion of an IKK subunit as defined herein, including synthetic peptides as described above. A non-immunogenic peptide portion of an IKK catalytic subunit can be made immunogenic by coupling the hapten to a carrier molecule such bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane, *supra*, 1988). It is recognized that, due to the apparently high amino acid sequence identity of the full length human IKKα and mouse CHUK, the amino acid sequences of IKKα polypeptides, as well as IKKβ polypeptides, likely are highly conserved among species, particularly among mammalian species. However, antibodies to highly conserved proteins have been raised successfully, for example, in chickens. Such a method can be used to obtain an antibody to an IKK subunit, if desired.

Particularly useful antibodies of the invention include antibodies that bind with the free, but not the complexed, form of an IKK subunit or, alternatively, with the complexed, but not free, form of an IKK subunit. Antibodies of the invention also include antibodies that bind with the 300 kDa IκB kinase complex or the 900 kDa IκB kinase complex or both. It should be recognized, however, that an antibody specific for the 300 kDa or 900 kDa IκB kinase complex need not recognize an IKK subunit epitope in order to be encompassed within the claimed invention, since, prior to the present disclosure, the 300 kDa and 900 kDa IKK complexes were not known (see DiDonato et al., Nature 388:548-554 (1997)).

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Antibodies of the invention that bind to an activated IKK but not to an inactive IKK, and, conversely, those that bind to an inactive form of the kinase but not to the activated form also are

5   particularly useful. For example, an IKK can be activated by phosphorylation of an IKK subunit and, therefore, an antibody that recognizes the phosphorylated form of the IKK, but that does not bind to the unphosphorylated form can be obtained. In addition, IKK

10 can be activated by release of a regulatory subunit and, therefore, an antibody that recognizes a form of the IKK complex that is not bound to the regulatory subunit can be obtained. Such antibodies are useful for identifying the presence of active IKK in a cell.

15   An anti-IKK antibody is useful, for example, for determining the presence or level of an IKK or of an IKK subunit in a tissue sample, which can be a lysate or a histological section. The identification of the presence or level of an IKK or an IKK subunit in the

20 sample can be made using well known immunoassay and immunohistochemical methods (Harlow and Lane, *supra*, 1988). An anti-IKK antibody also can be used to substantially purify an IκB kinase or an IKK subunit from a sample. In addition, an anti-IKK antibody can be used

25 in a screening assay to identify agents that alter the activity of an IκB kinase.

A kit incorporating an anti-IKK antibody, which can be specific for the active or inactive form of IκB kinase or can bind to an IKK complex or to an IKK

30 subunit, regardless of the activity state, can be particularly useful. Such a kit can contain, in addition to an anti-IKK antibody, a reaction cocktail that provides the proper conditions for performing the assay, control samples that contain known amounts of an IKK or

35 IKK subunit and, if desired, a second antibody specific

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for the anti-IKK antibody. Such an assay also should include a simple method for detecting the presence or amount of an IKK or an IKK subunit in a sample that is bound to the anti-IKK antibody.

5   A protein such as anti-IKK antibody, as well as an IKK subunit or a peptide portion thereof, can be labeled so as to be detectable using methods well known in the art (Hermanson, "Bioconjugate Techniques" (Academic Press 1996), which is incorporated herein by
10   reference; Harlow and Lane, 1988; chap. 9). For example, a protein can be labeled with various detectable moieties including a radiolabel, an enzyme, biotin or a fluorochrome. Reagents for labeling a protein such as an anti-IKK antibody can be included in a kit containing the
15   protein or can be purchased separately from a commercial source.

Following contact, for example, of a labeled antibody with a sample such as a tissue homogenate or a histological section of a tissue, specifically bound
20   labeled antibody can be identified by detecting the particular moiety. Alternatively, a labeled second antibody can be used to identify specific binding of an unlabeled anti-IKK antibody. A second antibody generally will be specific for the particular class of the first
25   antibody. For example, if an anti-IκB kinase antibody is of the IgG class, a second antibody will be an anti-IgG antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labeled using a detectable moiety as described above.
30   When a sample is labeled using a second antibody, the sample is first contacted with a first antibody, which is an anti-IKK antibody, then the sample is contacted with the labeled second antibody, which specifically binds to the anti-IKK antibody and results in a labeled sample.

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Methods for raising polyclonal antibodies, for
example, in a rabbit, goat, mouse or other mammal, are
well known in the art (see Example V). In addition,
monoclonal antibodies can be obtained using methods that
5   are well known and routine in the art (Harlow and Lane,
supra, 1988). Essentially, spleen cells from a mouse
immunized with an IKK complex or an IKK subunit or
peptide portion thereof can be fused to an appropriate
myeloma cell line such as SP/02 myeloma cells to produce
10   hybridoma cells. Cloned hybridoma cell lines can be
screened using a labeled IKK subunit to identify clones
that secrete anti-IKK monoclonal antibodies. Hybridomas
expressing anti-IKK monoclonal antibodies having a
desirable specificity and affinity can be isolated and
15   utilized as a continuous source of the antibodies, which
are useful, for example, for preparing standardized kits
as described above. Similarly, a recombinant phage that
expresses, for example, a single chain anti-IKK also
provides a monoclonal antibody that can used for
20   preparing standardized kits.

A monoclonal anti-IKK antibody can be used to
prepare anti-idiotypic antibodies, which present an
epitope that mimics the epitope recognized by the
monoclonal antibody used to prepare the anti-idiotypic
25   antibodies. Where the epitope to which the monoclonal
antibody includes, for example, a portion of the IKK
catalytic subunit kinase domain, the anti-idiotypic
antibody can act as a competitor of IκB and, therefore,
can be useful for reducing the level of phosphorylation
30   of IκB and, consequently, the activity of NF-κB.

The present invention further provides methods
of identifying an agent that can alter the association of
an IKK catalytic subunit with a second protein, which can
be an upstream activator, a downstream effector such as
35   IκB, an interacting regulatory protein of the IKK

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subunit, or an interacting subunit associated with the
300 kDa or 900 kDa IκB kinase complex. As used herein,
the term "associate" or "association," when used in
reference to an IKK subunit and a second protein means

5   that the IKK subunit and the second protein have a
binding affinity for each other such that they form a
bound complex *in vivo* or *in vitro*, including in a cell in
culture or in a reaction comprising substantially
purified reagents. For convenience, the term "bind" or

10   "interact" is used interchangeably with the term
"associate."

        The affinity of binding of an IKK subunit and a
second protein such as an IκB or another IKK subunit or
other subunit present in an IKK complex is characterized

15   in that it is sufficiently specific such that a bound
complex can form *in vivo* in a cell or can form *in vitro*
under appropriate conditions as disclosed herein. The
formation or dissociation of a bound complex can be
identified, for example, using the two hybrid assay or

20   demonstrating coimmunoprecipitation of the second protein
with the IKK subunit, as disclosed herein, or using other
well known methods such as equilibrium dialysis. Methods
for distinguishing the specific association of an IKK
subunit and a second protein from nonspecific binding to

25   the IKK subunit are known in the art and, generally,
include performing the appropriate control experiments to
demonstrate the absence of nonspecific protein binding.

        As used herein, the term "second protein"
refers to a protein that specifically associates with an

30   IKK subunit ("first protein"). Such a second protein is
exemplified herein by IκB proteins, including IκBα and
IκBβ, which are substrates for IκB kinase activity and
are downstream of the IκB kinase in a signal transduction
pathway that results in the regulated expression of a

35   gene. In addition, such second proteins are exemplified

by the proteins that, together with the IKK subunits, form a 300 kDa or 900 kDa IκB kinase complex, which coimmunoprecipitates using an anti-IKK antibody (see Example IV). Furthermore, since IKK subunits such as IKKα and IKKβ interact with each other to form homodimers or heterodimers, a second protein also can be a second IKK subunit, which can be the same as or different from the "first" protein.

Agents that alter the association of an IKK catalytic subunit and a second protein such as IκB protein or an IKK regulatory subunit can be extremely valuable, for example, for limiting excessive cytokine expression as occurs in an acute phase response by preventing the activation of NF-κB, thereby preventing NF-κB mediated induction of cytokine gene expression. Where, in a drug screening assay of the invention, the second protein is an IκB, the IKK subunit can be any protein involved in IκB kinase activity, including, for example, mouse CHUK (Connelly and Marcu, *supra*, 1995; GenBank Accession #12473), which, prior to the present disclosure, was not known to have the ability to associate with IκB or to have IκB kinase activity.

In addition, a second protein can be a protein that is upstream of IκB kinase in a signal transduction pathway and associates with the IKK complex, particularly with an IKK catalytic subunit of the IKK complex. Such a second protein, which can be an upstream activator of the IκB kinase, can be identified using routine methods for identifying protein-protein interactions as disclosed herein. Such second proteins can be, for example, MEKK1 or PKR or CKII, each of which has been reported to be involved in a pathway leading to phosphorylation of IκB and activation of NF-κB, but neither of which has the characteristics expected of the common IκB kinase present at the point where the various NF-κB activation pathways

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converge (see, for example, Lee et al., *supra*, 1997), or can be the NF-κB-inducing kinase (NIK), which reportedly is upstream from IKK in an NF-κB activation pathway (Regnier et al., *supra*, 1997; Malinin et al., <u>Nature</u>

5   385:540-544 (1997)).

         A second protein also can be a regulatory protein, which associates with an IKK catalytic subunit in an IKK complex, either constitutively as part of a 300 kDa or 900 kDa complex or in response to activation

10   of a pathway leading to IKK activation. Such a regulatory protein can inhibit or activate IKK activity depending, for example, on whether the regulatory protein is associated with IKK and whether the regulatory protein associates with an IKK catalytic subunit in a free form

15   or as part of an IKK complex. The regulatory protein also can be important for "docking" a catalytic IKK subunit to its substrate. The ability of a regulatory protein to associate with or dissociate from an IKK subunit or IKK complex can depend, for example, on the

20   relative phosphorylation state of the regulatory protein. It is recognized that an upstream activator of IKK also can interact with such a regulatory protein, thereby indirectly inhibiting or activating the IKK.

         As disclosed herein, two copurifying proteins

25   were isolated by ATP and IκB affinity chromatography and identified by SDS-PAGE (Example I). Partial amino acid sequences were determined and cDNA molecules encoding the proteins were obtained (see Examples I, II and III). One of the proteins has an apparent molecular mass of 85 kDa.

30   Expression in a cell of a cDNA molecule encoding the 85 kDa protein resulted in increased NF-κB activity following cytokine induction as compared to control cells, whereas expression of the antisense of this cDNA decreased the basal NF-κB activity in the cells and

35   prevented cytokine induction of NF-κB activity.

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Immunoprecipitation of the 85 kDa protein resulted in isolation of the IKK complex, the kinase activity of which was stimulated rapidly in response to TNF or to IL-1. Based on these functional analyses, the 85 kDa
5   protein was determined to be a component of the 900 kDa IκB kinase complex and has been designated IKKα (SEQ ID NO: 2). The second protein, which copurified with the 85 kDa IκB kinase, has an apparent molecular mass of 87 kDa and shares greater than 50% amino acid sequence identity
10   with IKKα and has been designated IKKβ (SEQ ID NO: 15).

The ability of the 85 kDa and 87 kDa IKK subunits to associate with other proteins such as a regulatory subunit as well as with IκB is suggested, for example, by the presence in the IκB kinase of two
15   different protein binding domains, a helix-loop-helix domain and a leucine zipper domain (see Connelly and Marcu, supra, 1995; see, also, Figure 3). While the leucine zipper motif mediates homotypic and heterotypic interactions between IKKα and IKKβ, the helix-loop-helix
20   motif serves as a binding site for regulatory proteins necessary for IκB kinase activation.

A screening assay of the invention provides a means to identify an agent that alters the association of an IKK complex or an IKK catalytic subunit with a second
25   protein such as the regulatory subunits discussed above. As used herein, the term "modulate" or "alter" when used in reference to the association of an IKK and a second protein, means that the affinity of the association is increased or decreased with respect to a steady state,
30   control level of association, i.e., in the absence of an agent. Agents that can alter the association of an IKK with a second protein can be useful for modulating the level of phosphorylation of IκB in a cell, which, in turn, modulates the activity of NF-κB in the cell and the
35   expression of a gene regulated by NF-κB. Such an agent

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can be, for example, an anti-idiotypic antibody as described above, which can inhibit the association of an IKK and IκB. A peptide portion of IκBα comprising amino acids 32 to 36, but containing substitutions for Ser-32

5   and Ser-36, is another example of such an agent, since the peptide can compete with IκBα binding to IKK, as is the corresponding peptide of IκBβ.

A screening assay of the invention also is useful for identifying agents that directly alter the

10   activity of an IKK. While such an agent can act, for example, by altering the association of an IKK complex or IKK catalytic subunit with a second protein, the agent also can act directly as a specific activator or inhibitor of IKK activity. Specific protein

15   kinase inhibitors include, for example, staurosporin, the heat stable inhibitor of cAMP-dependent protein kinase, and the MLCK inhibitor, which are known in the art and commercially available. A library of molecules based, generally, on such inhibitors or on ATP or adenosine can

20   be screened using an assay of the invention to obtain agents that desirably modulate the activity of an IKK complex or an IKK subunit.

As disclosed herein, IKK activity can be measured by identifying phosphorylation, for example, of

25   IκBα, either directly or using an antibody specific for the Ser-32 and Ser-36 phosphorylated form of IκBα. An antibody that binds to IκBα that is phosphorylated on Ser-32, for example, can be purchased from a commercial source (New England Biolabs; Beverly MA). Cultured cells

30   can be exposed to various agents suspected of having the ability to directly alter IKK activity, then aliquots of the cells either are collected or are treated with a proinflammatory stimulus such as a cytokine, and collected. The collected cells are lysed and the kinase

35   is immunoprecipitated using an anti-IKK antibody. A

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substrate such as IκBα or IκBβ is added to the
immunocomplex and the ability of the IKK to phosphorylate
the substrate is determined as described above. If
desired, the anti-IKK antibody first can be coated onto a
5   plastic surface such as in 96 well plates, then the cell
lysate is added to the wells under conditions that allow
binding of IKK by the antibody. Following washing of the
wells, IKK activity is measured as described above. Such
a method is extremely rapid and provides the additional
10   advantage that it can be automated for high through-put
assays.

        A screening assay of the invention is
particularly useful to identify, from among a diverse
population of molecules, those agents that modulate the
15   association of an IKK complex or an IKK catalytic subunit
and another protein (referred to herein as a "second
protein") or that directly alter the activity of IKK.
Methods for producing libraries containing diverse
populations of molecules, including chemical or
20   biological molecules such as simple or complex organic
molecules, peptides, proteins, peptidomimetics,
glycoproteins, lipoproteins, polynucleotides, and the
like, are well known in the art (Huse, U.S. Patent No.
5,264,563, issued November 23, 1993; Blondelle et al.,
25   Trends Anal. Chem. 14:83-92 (1995); York et al., Science
274:1520-1522 (1996); Gold et al., Proc. Natl. Acad.
Sci., USA 94:59-64 (1997); Gold, U.S. Patent No.
5,270,163, issued December 14, 1993). Such libraries
also can be obtained from commercial sources.

30   Since libraries of diverse molecules can
contain as many as 10^{14} to 10^{15} different molecules, a
screening assay of the invention provides a simple means
for identifying those agents in the library that can
modulate the association of an IKK and a second protein
35   or can alter the activity of an IKK. In particular, a

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screening assay of the invention can be automated, which
allows for high through-put screening of randomly
designed libraries of agents to identify those particular
agents that can modulate the ability of an IKK and a
5   second protein to associate or that alter the activity of
the IKK.

A drug screening assay of the invention
utilizes an IKK complex, which can be isolated as
disclosed herein; or an IKK subunit, which can be
10   expressed, for example, from a nucleic acid molecule
encoding the amino acid sequence shown in SEQ ID NO: 2 or
in SEQ ID NO: 15; or can be purified as disclosed herein;
or can utilize an IKK subunit fusion protein such as an
IKKα-glutathione-S-transferase (GST) or IKKβ-histidine$_6$
15   (HIS6) fusion protein, wherein the GST or HIS6 is linked
to the IKK subunit and comprises a tag (see Example VI).
The IKK or IKK subunit fusion protein is characterized,
in part, by having an affinity for a solid substrate as
well as having the ability to specifically associate with
20   an appropriate second protein such as an IκB protein.
For example, when an IKK catalytic subunit is used in a
screening assay, the solid substrate can contain a
covalently attached anti-IKK antibody, provided that the
antibody binds the IKK subunit without interfering with
25   the ability of the IKK subunit to associate with the
second protein. Where an IKKα-GST fusion protein, for
example, is used in such a screening assay, the solid
substrate can contain covalently attached glutathione,
which is bound by the GST tag component of the fusion
30   protein. If desired, the IKK subunit or IKK subunit
fusion protein can be part of an IKK complex in a drug
screening assay of the invention.

A drug screening assay to identify an agent
that alters the association of an IKK complex or an IKK
35   subunit and a second protein can be performed by

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allowing, for example, the IKK complex or IKK subunit, which can be a fusion protein, to bind to the solid support, then adding the second protein, which can be an IκB such as IκBα, and an agent to be tested, under

5   conditions suitable for the association of the IKK and IκBα in the absence of a drug (see Example VI). As appropriate, the IKK can be activated or inactivated as disclosed herein and, typically, the IKK or the second protein is detectably labeled so as to facilitate

10 identification of the association. Control reactions, which contain or lack either, the IKK component, or the IκB protein, or the agent, or which substitute the IκB protein with a second protein that is known not to associate specifically with the IKK, also are performed.

15 Following incubation of the reaction mixture, the amount of IκBα specifically bound to the IKK in the presence of an agent can be determined and compared to the amount of binding in the absence of the agent so that agents that modulate the association can be identified.

20   An IKK subunit such as IKKα or IKKβ used in a screening assay can be detectably labeled with a radionuclide, a fluorescent label, an enzyme, a peptide epitope or other such moiety, which facilitates a determination of the amount of association in a reaction.

25 By comparing the amount of specific binding of an IKK subunit or an IKK complex and IκB in the presence of an agent as compared to the control level of binding, an agent that increases or decreases the binding of the IKK and the IκB can be identified. In comparison, where a

30 drug screening assay is used to identify an agent that alters the activity of an IKK, the detectable label can be, for example, γ-^{32}P-ATP, and the amount of ^{32}P-IκB can be detected as a measure of IKK activity. Thus, the drug screening assay provides a rapid and simple method for

35 selecting agents that desirably alter the association of an IKK and a second protein such as an IκB or for

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altering the activity of an IKK. Such agents can be
useful, for example, for modulating the activity of NF-κB
in a cell and, therefore, can be useful as medicaments
for the treatment of a pathology due, at least in part,
5 to aberrant NF-κB activity.

The method for performing a drug screening
assay as disclosed herein also provides a research tool
for identifying a target of a drug that is or can be used
therapeutically to ameliorate an undesirable inflammatory
10 or immune response, but for which the target of the drug
is not known. Cytokine restraining agents, for example,
are a class of agents that can alter the level of
cytokine expression (U.S. Patent No. 5,420,109, issued
May 30, 1995) and can be used to treat various
15 pathologies, including patho-immunogenic diseases such as
rheumatoid arthritis and those induced by exposure to
bacterial endotoxin such as occur in septic shock (see,
also, WO96/27386, published September 12, 1996).

The specific cellular target upon which a
20 cytokine restraining agent acts has not been reported.
However, the myriad of pathologic effects ameliorated by
such agents are similar to various pathologies associated
with aberrant NF-κB activity, suggesting that cytokine
restraining agents may target an effector molecule in a
25 NF-κB signal transduction pathway. Thus, one potential
target of a cytokine restraining agent can be an IκB
kinase, particularly an IKK catalytic subunit of the
kinase. Accordingly, a screening assay of the invention
can be used to determine whether a cytokine restraining
30 agent alters the activity of IκB kinase or alters the
association of an IKK and a second protein such as IκB.
If it is determined that a cytokine restraining agent has
such an effect, the screening assay then can be used to
screen a library of cytokine regulatory agents to

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identify those having desirable characteristics, such as those having the highest affinity for the IKK.

The invention also provides a method of obtaining an isolated IKK complex or an IKK catalytic

5   subunit. For example, a 300 kDa or a 900 kDa IKK complex, comprising an IKKα subunit can be isolated from a sample by immunoprecipitation using an anti-IKKα antibody or by tagging the IKKα and using an antibody specific for the tag (see Examples III and IV). In

10 addition, an IKK catalytic subunit can be isolated from a sample by 1) incubating the sample containing the IKK subunit with ATP, which is immobilized on a matrix, under conditions suitable for binding of the IKK subunit to the ATP; 2) obtaining from the immobilized ATP a fraction of

15 the sample containing the IKK subunit; 3) incubating the fraction containing the IKK subunit with an IκB, which is immobilized on a matrix, under conditions suitable for binding of the IKK subunit to the IκB; and 4) obtaining from the immobilized IκB an isolated IKK catalytic

20 subunit. Such a method of isolating an IKK subunit is exemplified herein by the use of ATP affinity chromatography and IκBα affinity chromatography to isolate IKKα or IKKβ from a sample of HeLa cells (see Example I).

25 The skilled artisan will recognize that a ligand such as ATP or an IκB or an anti-IKK antibody also can be immobilized on various other matrices, including, for example, on magnetic beads, which provide a rapid and simple method of obtaining a fraction containing an ATP-

30 or an IκB-bound IKK complex or IKK subunit or an anti-IκB kinase-bound IKK from the remainder of the sample. Methods for immobilizing a ligand such as ATP or an IκB or an antibody are well known in the art (Haystead et al., Eur. J. Biochem. 214:459-467 (1993), which is

35 incorporated herein by reference; see, also, Hermanson,

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supra, 1996). Similarly, the artisan will recognize that a sample containing an IKK complex or an IKK subunit can be a cell, tissue or organ sample, which is obtained from an animal, including a mammal such as a human, and

5   prepared as a lysate; or can be a bacterial, insect, yeast or mammalian cell lysate, in which an IKK catalytic subunit is expressed from a recombinant nucleic acid molecule. As disclosed herein, a recombinantly expressed IKKα or IKKβ such as a tagged IKKα or IKKβ associates

10 into an active 300 kDa and 900 kDa IKK complex (see Examples III and IV).

The invention also provides a method of identifying a second protein that associates with an IKK complex, particularly with an IKK subunit. A

15 transcription activation assay such as the yeast two hybrid system is particularly useful for the identification of protein-protein interactions (Fields and Song, Nature 340:245-246 (1989), which is incorporated herein by reference). In addition, the two

20 hybrid assay is useful for the manipulation of protein-protein interaction and, therefore, also is useful in a screening assay to identify agents that modulate the specific interaction.

A transcription activation assay such as the

25 two hybrid assay also can be performed in mammalian cells (Fearon et al., Proc. Natl. Acad. Sci., USA 89:7958-7962 (1992), which is incorporated herein by reference). However, the yeast two hybrid system provides a particularly useful assay due to the ease of working with

30 yeast and the speed with which the assay can be performed. Thus, the invention also provides methods of identifying proteins that can interact with an IKK subunit, including proteins that can act as upstream activators or downstream effectors of IKK activity in a

35 signal transduction pathway mediated by the IKK or

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proteins that bind to and regulate the activity of the
IKK. Such proteins that interact with an IKK catalytic
subunit can be involved, for example, in tissue specific
regulation of NF-κB activation or constitutive NF-κB
5   activation and consequent gene expression.

       The conceptual basis for a transcription
activation assay is predicated on the modular nature of
transcription factors, which consist of functionally
separable DNA-binding and trans-activation domains. When
10   expressed as separate proteins, these two domains fail to
mediate gene transcription. However, the ability to
activate transcription can be restored if the DNA-binding
domain and the trans-activation domain are bridged
together through a protein-protein interaction. These
15   domains can be bridged, for example, by expressing the
DNA-binding domain and trans-activation domain as fusion
proteins (hybrids), where the proteins that are appended
to these domains can interact with each other. The
protein-protein interaction of the hybrids can bring the
20   DNA-binding and trans-activation domains together to
create a transcriptionally competent complex.

       One adaptation of the transcription activation
assay, the yeast two hybrid system, uses *S. cerevisiae* as
a host cell for vectors that express the hybrid proteins.
25   For example, a yeast host cell containing a reporter lacZ
gene linked to a LexA operator sequence can be used to
identify specific interactions between an IKK subunit and
a second protein, where the DNA-binding domain is the
LexA binding domain, which binds the LexA promoter, and
30   the trans-activation domain is the B42 acidic region.
When the LexA domain is bridged to the B42
transactivation domain through the interaction of the IKK
subunit with a second protein, which can be expressed,
for example, from a cDNA library, transcription of the
35   reporter lacZ gene is activated. In this way, proteins

that interact with the IKK subunit can be identified and their role in a signal transduction pathway mediated by the IKK can be elucidated. Such second proteins can include additional subunits comprising the 300 kDa or
5 900 kDa IKK complex.

In addition to identifying proteins that were not previously known to interact with an IKK, particularly with an IKKα or IKKβ subunit, a transcription activation assay such as the yeast two
10 hybrid system also is useful as a screening assay to identify agents that alter association of an IKK subunit and a second protein known to bind the IKK. Thus, as described above for *in vitro* screening assays, a transcription activation assay can be used to screen a
15 panel of agents to identify those agents particularly useful for altering the association of an IKK subunit and a second protein in a cell. Such agents can be identified by detecting an altered level of transcription of a reporter gene, as described above, as compared to
20 the level of transcription in the absence of the agent. For example, an agent that increases the interaction between an IKK subunit and IκB can be identified by an increased level of transcription of the reporter gene as compared to the control level of transcription in the
25 absence of the agent. Such a method is particularly useful because it identifies an agent that alters the association of an IKK subunit and a second protein in a living cell.

In some cases, an agent may not be able to
30 cross the yeast cell wall and, therefore, cannot enter the yeast cell to alter a protein-protein interaction. The use of yeast spheroplasts, which are yeast cells that lack a cell wall, can circumvent this problem (Smith and Corcoran, In Current Protocols in Molecular Biology (ed.
35 Ausubel et al.; Green Publ., NY 1989), which is

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incorporated herein by reference). In addition, an
agent, upon entering a cell, may require "activation" by
a cellular mechanism that may not be present in yeast.
Activation of an agent can include, for example,
5   metabolic processing of the agent or a modification such
as phosphorylation of the agent, which can be necessary
to confer activity upon the agent. In this case, a
mammalian cell line can be used to screen a panel of
agents (Fearon et al., *supra*, 1992).

10   An agent that alters the catalytic activity of
an IKK or that alters the association of an IKK subunit
or IKK complex and a second protein such as an IκB or an
IKK regulatory subunit or an upstream activator of an IKK
can be useful as a drug to reduce the severity of a
15 pathology characterized by aberrant NF-κB activity. For
example, a drug that increases the activity of an IKK or
that increases the affinity of an IKK catalytic subunit
and IκBα can increase the amount of IκBα phosphorylated
on Ser-32 or Ser-36 and, therefore, increase the amount
20 of active NF-κB and the expression of a gene regulated by
NF-κB, since the drug will increase the level of
phosphorylated IκBα in the cell, thereby allowing NF-κB
translocation to the nucleus. In contrast, a drug that
decreases or inhibits the catalytic activity of an IKK or
25 the association of an IKK catalytic subunit and IκBα can
be useful where it is desirable to decrease the level of
active NF-κB in a cell and the expression of a gene
induced by activated NF-κB. It should be recognized that
an antisense IKK subunit molecule of the invention also
30 can be used to decrease IKK activity in a cell by
reducing or inhibiting expression of the IKK subunit or
by reducing or inhibiting its responsiveness to an
inducing agent such as TNFα, Il-1 or phorbol ester (see
Example II). Accordingly, the invention also provides
35 methods of treating an individual suffering from a
pathology characterized by aberrant NF-κB activity by

administering to the individual an agent that modulates
the catalytic activity of an IKK or that alters the
association of an IKK subunit and a second protein such
as IκB or a subunit of a 300 kDa or 900 kDa IKK complex
5   that interacts with the IKK subunit.

An agent that decreases the activity of an IKK
or otherwise decreases the amount of IκB phosphorylation
in a cell can reduce or inhibit NF-κB mediated gene
10   expression, including, for example, the expression of
proinflammatory molecules such as cytokines and other
biological effectors involved in an inflammatory, immune
or acute phase response. The ability to reduce or
inhibit such gene expression can be particularly valuable
15   for treating various pathological conditions such as
rheumatoid arthritis, asthma and septic shock, which are
characterized or exacerbated by the expression of such
proinflammatory molecules.

Glucocorticoids are potent anti-inflammatory
20   and immunosuppressive agents that are used clinically to
treat various pathologic conditions, including autoimmune
diseases such as rheumatoid arthritis, systemic lupus
erythematosis and asthma. Glucocorticoids suppress the
immune and inflammatory responses, at least in part, by
25   increasing the rate of IκBα synthesis, resulting in
increased cellular levels of IκBα, which bind to and
inactivate NF-κB (Scheinman et al., <u>Science</u> 270:283-286
(1995); Auphan et al., <u>Science</u> 270:286-290 (1995)).
Thus, glucocorticoids suppress NF-κB mediated expression
30   of genes encoding, for example, cytokines, thereby
suppressing the immune, inflammatory and acute phase
responses. However, glucocorticoids and glucocorticoid-
like steroids also are produced physiologically and are
required for normal growth and development.
35   Unfortunately, prolonged treatment of an individual with
higher than physiological amounts of glucocorticoids

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produces clinically undesirable side effects. Thus, the
use of an agent that alters the activity of an IKK or
that alters the association of an IKK complex or IKK
subunit and a second protein, as identified using a
5   method of the invention, can provide a means for
selectively altering NF-κB activity without producing
some of the undesirable side effects associated with
glucocorticoid treatment.

            Inappropriate regulation of Rel/NF-κB
10   transcription factors is associated with various human
diseases. For example, many viruses, including human
immunodeficiency virus-1 (HIV-1), herpes simplex virus-1
(HSV-1) and cytomegalovirus (CMV) contain genes regulated
by a κB regulatory element and these viruses, upon
15   infecting a cell, utilize cellular Rel/NF-κB
transcription factors to mediate viral gene expression
(Siebenlist et al., *supra*, 1994). Tat-mediated
transcription from the HIV-1 enhancer, for example, is
decreased if the NF-κB and SP1 binding sites are deleted
20   from the enhancer/promotor region, indicating that Tat
interacts with NF-κB, SP1 or other transcription factors
bound at this site to stimulate transcription (Roulston
et al., <u>Microbiol. Rev.</u> 59:481-505 (1995)). In addition,
chronic HIV-1 infection, and progression to AIDS, is
25   associated with the development of constitutive NF-κB DNA
binding activity in myeloid cells (Roulston et al.,
supra, 1995). Thus, a positive autoregulatory loop is
formed, whereby HIV-1 infection results in constitutively
active NF-κB, which induces expression of HIV-1 genes
30   (Baeuerle and Baltimore, <u>Cell</u> 87:13-20 (1996).
Constitutive NF-κB activation also may protect cells
against apoptosis, preventing clearance of virus-infected
cells by the immune system (Liu et al., *supra*, 1996).

            An agent that decreases the activity of an IKK
35   or that alters the association of an IKK and a second

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protein such that IκB phosphorylation is decreased can be useful for reducing the severity of a viral infection such as HIV-1 infection in an individual by providing increased levels of unphosphorylated IκB in virus-
5 infected cells. The unphosphorylated IκB then can bind to NF-κB in the cell, thereby preventing nuclear translocation of the NF-κB and viral gene expression. In this way, the rate of expansion of the virus population can be limited, thereby providing a therapeutic advantage
10 to the individual.

In addition, the decreased level of NF-κB activity may allow the virus-infected cell to undergo apoptosis, resulting in a decrease in the viral load in the individual. As such, it can be particularly useful
15 to treat virus-infected cells *ex vivo* with an agent identified using a method of the invention. For example, peripheral blood mononuclear cells (PBMCs) can be collected from an HIV-1 infected individual and treated in culture with an agent that decreases the activity of
20 an IKK or alters the association of an IKK complex or an IKK catalytic subunit with an IκB. Such a treatment can be useful to purge the PBMCs of the virus-infected cells by allowing apoptosis to proceed. The purged population of PBMCs then can be expanded, if desired, and
25 readministered to the individual.

Rel/NF-κB proteins also are involved in a number of different types of cancer. For example, the adhesion of cancer cells to endothelial cells is increased due to treatment of the cancer cells with IL-1,
30 suggesting that NF-κB induced the expression of cell adhesion molecules, which mediated adherence of the tumor cells to the endothelial cells; agents such as aspirin, which decrease NF-κB activity, blocked the adhesion by inhibiting expression of the cell adhesion molecules
35 (Tozawa et al., Cancer Res. 55:4162-4167 (1995)). These

results indicate that an agent that decreases the activity of an IKK or that decrease the association of an IKK and IκB or of an IKK subunit and a second protein, for example, a second protein present in an IKK complex,

5   can be useful for reducing the likelihood of metastasis of a tumor in an individual.

As discussed above for virus-infected cells, constitutive NF-κB activation also may protect tumor cells against programmed cell death as well as apoptosis

10 induced by chemotherapeutic agents (Liu et al., *supra*, 1996; Baeuerle and Baltimore, Cell 87:13-20 (1996)). Thus, an agent that decreases IKK activity or that decreases the association of IKK and IκB also can be useful for allowing programmed cell death to occur in a

15 tumor cell by increasing the level of unphosphorylated IκB, which can bind NF-κB and decrease the level of active NF-κB in the tumor cell.

The following examples are intended to illustrate but not limit the present invention.

20   **EXAMPLE I**

IDENTIFICATION AND CHARACTERIZATION OF
A HUMAN IκB KINASE COMPLEX AND IKK SUBUNITS

This example provides a method for identifying and isolating a cytokine responsive protein kinase

25 complex that phosphorylates IκB, which regulates NF-κB activity, and catalytic subunits of the protein kinase complex.

A. Kinase assays:

Kinase assays were performed using GST fusion

30 proteins containing amino acid residues 1 to 54 of IκB. The fusion proteins were linked to glutathione SEPHAROSE and the beads were used directly in the assays.   At

earlier stages in the purification of the IKK activity, the beads were washed prior to loading onto the gel to minimize contributions from other proteins. In some of the later characterization of highly purified material,

5   soluble fusion protein was used.

Three distinct substrates for the IKK activity were used: 1) substrate "WT" contained amino acid residues 1 to 54 of IκBα; 2) substrate "AA" contained amino acid residues 1 to 54 of IκBα, except that Ser-32

10   (S32) and S36 were replaced with Ala-32 (A32) and A36, respectively; and 3) substrate "TT" contained amino acid residues 1 to 54 of IκBα, except that S32 and S36 were replaced with Thr-32 (T32) and T36, respectively (DiDonato et al., Mol. Cell. Biol. 16:1295-1304 (1996)).

15   Each substrate was expressed as a GST fusion protein. The physiologic, inducible IκB kinase is specific for S32 and S36 (WT) in IκBα, but does not recognize the TT or AA mutants (DiDonato et al., Mol. Cell. Biol. 16:1295-1304 (1996)).

20   Kinase assays were carried out in 20 mM HEPES (pH 7.5-7.6), 20 mM β-glycerophosphate (β-GP), 10 mM $MgCl_2$, 10 mM PNPP, 100 μM Na_3VO_4, 2 mM dithiothreitol (DTT), 20 μM ATP, 10 μg/ml aprotinin. NaCl concentration was 150-200 mM and the assays were carried out at 30°C

25   for 30 min. Fractionation was performed by SDS-PAGE, followed by quantitation by phosphoimager analysis.

B. Purification of IKK complex and IKK subunits:

The protein purification buffer (Buffer A) consisted of 20 mM Tris (pH 7.6, measured at RT), 20 mM

30   NaF, 20 mM β-GP, 1 mM PNPP, 500 μM Na_3VO_4, 2 mM DTT, 2.5 mM metabisulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, and 10% glycerol. Brij-35 was added as indicated. Cell lysis buffer was Buffer A containing an

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additional 19 mM PNPP, 20 mM β-GP and 500 μM Na₃VO₄, and 20 μg/ml aprotinin, 2.5 μg/ml leupeptin, 8.3 μg/ml bestatin, 1.7 μg/ml pepstatin.

5 Purification was performed using 5 to 130 liters of HeLa S3 cells. For illustration, the procedure for a 15 liter preparation is presented. All purification steps were performed in a cold room at 4°C.

In order to activate the IKK, cells were stimulated with TNFα prior to purification. TNFα was 10 either recombinant TNFα, which was purchased from R&D Systems and used at 20 ng/ml, or HIS6-tagged TNFα, which was expressed and partially purified from *E. coli* and used at 5 μg/ml. TNFα-induced HeLa S3 cell killing activity assays were performed in the presence of 15 cycloheximide and indicated that the partially purified HIS6-tagged TNFα had approximately one-tenth the activity of the commercial TNFα.

Fifteen liters of HeLa S3 cells were grown in suspension in high glucose Dulbecco's modified Eagle's 20 medium supplemented with 10% calf serum, 2 mg/ml L-glutamine, 100 U/ml penicillin/streptomycin, 0.11 mg/ml sodium pyruvate, and 1X nonessential amino acids (Irvine Scientific; Irvine CA). Cell density was approximately 5 x 10⁵ cells/ml at the time of collection. Cells were 25 concentrated 10-fold by centrifugation. stimulated for 5 min with TNFα at 37°C, then diluted with 2.5 volumes of ice cold phosphate buffered saline (PBS) containing 50 mM NaF and pelletted at 2000 x g. The cell pellet was washed once with ice cold PBS/50 mM NaF, then suspended 30 in lysis buffer, quick frozen in liquid nitrogen and stored at ¯80°C.

For purification of IκB kinase, cells were thawed and cytoplasmic extract prepared. Lysis was

achieved by 40 strokes in an all glass Dounce homogenizer
(pestle A) in lysis buffer containing 0.05% NP-40 on ice.
The homogenate was centrifuged at 12,000 rpm for 19 min
in a Beckman SS34 rotor at 4°C.

5   Supernatant was collected and centrifuged at
38,000 rpm for 80 min in a Beckman 50.1 Ti rotor at 4°C.
The supernatant (S100 fraction) was quick frozen in
liquid nitrogen and stored at -80°C. Small aliquots of
S100 material, prepared from either unstimulated HeLa
10   cells or from TNFα stimulated cells, were purified in a
single passage over a SUPEROSE 6 gel filtration column
(1.0 x 30 cm; Pharmacia; Uppsalla Sweden) equilibrated in
Buffer A containing 0.1% Brij-35 and 300 mM NaCl and
eluted at a flow rate of 0.3 ml/min. 0.6 ml fractions
15   were collected and kinase assays were performed on an
aliquot of each fraction. The high molecular weight
material (fractions 16-20) contained TNFα-inducible IKK
activity, which is specific for the WT substrate.

         110 ml of S100 material (900 mg of protein;
20   Bio-Rad Protein Assay) was pumped onto a Q-SEPHAROSE FAST
FLOW column (56 ml bed volume, 2.6 cm ID) equilibrated at
2 ml/min with Buffer A containing 0.1% Brij-35. After
the sample was loaded, the column was washed with 100 ml
of Buffer A containing 0.1% Brij-35 and 100 mM NaCl, then
25   a linear NaCl gradient was run from 100-300 mM. The
gradient volume was 500 ml and the flow rate was
2 ml/min. Ten ml fractions were collected and the kinase
assay was performed on those fractions that eluted during
the gradient. Fractions corresponding to the
30   TNFα-inducible IKK activity (fractions 30-42; i.e., 20-32
of the gradient portion) were pooled. The pooled
material contained 40 mg of protein.

         The pooled material was diluted to 390 ml by
addition of Buffer A containing 0.1 % Brij-35 and loaded

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onto a pre-equilibrated 5 ml HITRAP Q column (Pharmacia)
at a flow rate of 4 ml/min. Following sample loading,
the column was washed with 20 ml of Buffer A containing
0.1 % Brij-35. The protein was eluted at 1 ml/min
5   isocratically in Buffer A containing 0.1 % Brij-35 and
300 mM NaCl and 1 ml fractions were collected. Protein-
containing fractions were identified using the BioRad
assay and were collected and pooled to yield 4 ml of
solution. Previously performed control experiments
10 demonstrated that the IKK activity directly correlated
with protein concentration.

The pooled material was diluted 1:1 with ATP
column buffer (20 mM HEPES (pH 7.3), 50 mM β-GP, 60 mM
$MgCl_2$, 1 mM Na_2VO_4, 1.5 mM EGTA, 1 mM DTT, 10 $\mu g/ml$
15 aprotinin), then passed 4 times over a γ-ATP affinity
column having 4 ml bed volume (Haystead et al., *supra*,
1993); the column had been prewashed with 2 M NaCl, 0.25%
Brij-35 and equilibrated with 10 bed volumes of ATP
column buffer containing 0.05% Brij-35 at a flow rate of
20 0.5 ml/min. Following loading of the sample, the column
was washed with 10 ml of ATP column buffer containing
0.05% Brij-35, then with 10 ml ATP column buffer
containing 0.05% Brij-35 and 250 mM NaCl.

Bound material was eluted in 10 ml of ATP
25 column buffer containing 0.05 % Brij-35, 250 mM NaCl and
10 mM ATP (elution buffer).   Elution was performed by
passing 5 ml of elution buffer through the column,
allowing the column to incubate, capped, for 20 min, then
passing an additional 5 ml of elution buffer through the
30 column. The samples were pooled to yield 10 ml.

The 10 ml pooled sample from the ATP column was
diluted with 30 ml Buffer A containing 0.1 % Brij-35 and
loaded onto a 1 ml HITRAP Q column (Pharmacia) at
1 ml/min. The column was eluted at 0.4 ml/min with

Buffer A containing 0.1 % Brij-35 and 300 mM NaCl.
0.2 ml fractions were collected and the four protein-
containing fractions were pooled (0.5 mg). The pooled
material was concentrated to 200 μl on a 10K NANOSEP
5   concentrator (Pall/Filtron) and loaded onto a SUPEROSE 6
gel filtration column (1.0 x 30 cm). The SUPEROSE 6
column was equilibrated in Buffer A containing 0.1 %
Brij-35 and 300 mM NaCl and run at a flow rate of 0.3
ml/min; 0.6 ml fractions were collected. Fractions 17,
10   18 and 19 contained kinase activity.

        Based on silver stained SDS-PAGE gels, the
final purified material consisted of approximately 20 μg
to 40 μg of total protein, of which approximately 2 μg
corresponded to the 85 kDa band, later designated IKKα
15   (see Example II). A second band migrating at 87 kDa was
later designated IKKβ (see Example III). The total time
from the thawing of the S100 material until the
collection of fractions from the gel filtration column
was 24 hours.

20   C. Confirmation of IKK purification:

        Since the 85 kDa IKKα band identified by the
kinase assay following the above procedure contained only
about 10% of the total purified protein, three additional
criteria were used to confirm that the identified band
25   was an intrinsic component of the IKK complex.

        In one procedure, the elution profile of the
SUPEROSE 6 column was analyzed by silver stained
8% SDS-PAGE gels, then compared to the kinase activity
profile. For this analysis, 0.3 ml fractions were
30   collected from the SUPEROSE 6 column, then separated by
8% SDS-PAGE and silver stained. This comparison
confirmed that a single band of 85 kDa correlated
precisely with the elution of IKK activity.

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In a second procedure, the IKK activity was further purified on a substrate affinity column at 4°C. A GST fusion protein was prepared containing the A32/A36 1 to 54 amino acid sequence of IκBα repeated 8 times

5   (GST-(8X-AA)). The GST-(8X-AA) then was covalently linked to a CNBr activated SEPHAROSE 4B resin to produce the substrate affinity resin.

IKK-containing material was diluted into Buffer A to yield a final concentration of 70 mM NaCl,

10 0.025% Brij-35, then added to the substrate affinity resin at a ratio of 4:1 (solution:swollen beads). The resin was suspended and the mixture rotated gently overnight in a small column at 4°C. The resin was allowed to settle for 30 min, then the column was eluted

15 by gravity. The column was washed with 4 bed volumes Buffer A containing 0.02% Brij-35, then the resin was suspended with 1.1 bed volumes of Buffer A containing 600 mM NaCl and 0.1 % Brij-35. The resin was allowed to settle for 40 min, then gravity elution was performed.

20 The column was washed with an additional 1.1 bed volumes of Buffer A containing 600 mM NaCl and 0.1 % Brij-35 and the two fractions were pooled.

The IκBα substrate affinity column was used for two separate experiments. In one experiment, the

25 material that eluted from the final SUPEROSE 6 column was further purified on the IκBα substrate affinity column. In the second experiment, material obtained after the initial Q-SEPHAROSE column was purified on the IκBα substrate affinity column. The Q-SEPHAROSE bound

30 fraction then was further purified on the ATP column and the SUPEROSE 6 column (see above).

Analysis of the purified material from these two experiments by silver stained SDS-PAGE gels revealed different protein profiles. However, comparison of these

profiles revealed only two bands common to both
preparations, one of which was confirmed to be the same
85 kDa IKKα band that was identified by the SUPEROSE 6
profile analysis and cofractionated with IκB kinase
5   activity. The other band, which was 87 kDa in size,
later was identified as IKKβ. In several different
experiments, the 85 kDa protein and 87 kDa protein were
specifically purified by the substrate affinity column in
what appeared to be an equimolar ratio.

10   In a third procedure, purified IKK was treated
with excess phosphatase, which inactivates the IKK, then
reactivated by addition of a semi-purified HeLa extract.
Phosphatase inactivation was performed by adding excess
protein phosphatase 2A catalytic domain (PP2A) to
15 purified IκB kinase in 50 mM Tris (pH 7.6), 50 mM NaCl,
1 mM $MgCl_2$, then equilibrating the reaction for 60 min at
30°C. 1.25 μM okadaic acid was added to completely
inactivate the phosphatase and the phosphatase
inactivated material was used in standard kinase assays
20 and to perform the reactivation and phosphorylation
procedure.

          Cytoplasmic extract was prepared using HeLa S3
cells. The cells were stimulated with TNFα for 5 min,
then harvested in lysis buffer containing 0.1 % NP-40 and
25 0.15 M NaCl. Reactivation was performed at 30°C in
kinase buffer for 60 min in the absence of (γ-^{32}P)ATP.
Samples containing only cold ATP were used for kinase
activity assays. Reactivation by the HeLa cell extract
was performed in the presence of (γ-^{32}P)ATP, then the
30 sample was separated by 8% SDS-PAGE and examined by
autoradiography. A band of approximately 86 kDa was
phosphorylated in the reactivated material and,
associated with the reactivation procedure, was
restoration of the IKK activity.

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D. Partial amino acid sequences of IKKα and IKKβ

            Following SDS-PAGE as described above, the
85 kDa IKKα and 87 kDa IKKβ bands were excised from the
gel and submitted for internal peptide sequencing
5   analysis. From the IKKα polypeptide, the sequences of
two proteolytic fragments were identified, as follows:
KIIDLLPK (SEQ ID NO: 3) and KHR(D/A)LKPENIVLQDVG(P/G)K
(SEQ ID NO: 4). Where a residue could not be
unambiguously determined, an "X" was used to indicate no
10   amino acid could be determined and parentheses were used
to delimit amino acids that could not be distinguished.
Since Lys-C protease was used to digest the protein, the
presence of lysine residues at the N-termini of the
peptides was inferred. From the 87 kDa IKKβ band, the
15   sequences of five proteolytic fragments were determined
(see Figure 3, underlined; see, also, Example III).

EXAMPLE II
IDENTIFICATION AND CHARACTERIZATION
OF A FULL LENGTH HUMAN IKKα SUBUNIT

20   This example provides methods for isolating a
nucleic acid molecule encoding the IKKα subunit and for
characterizing the functional activity of the subunit.

A. Cloning of cDNA encoding human IKKα:

            Degenerate oligonucleotide (length) sequences
25   of the amino acid sequences of two peptide fragments (SEQ
ID NOS: 3 and 4) of the IKKα (see Figure 1) were searched
in the GenBank DNA sequence database. This search
revealed that nucleotide sequences encoding both peptide
fragments were present in a partial cDNA encoding a
30   portion of a protein designated human CHUK (GenBank
Accession #U22512; Connelly and Marcu, *supra*, 1995).

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Based on the human CHUK cDNA sequence, PCR
primers were prepared corresponding to the 5'-terminus
(5'-CCC**CATATG**TACCAGCATCGGGAA-3'; SEQ ID NO: 5) and
3'-terminus (3'-CCC**CTCGA**GTTCTGTTAACCAACT-5'; SEQ ID

5   NO: 6). SEQ ID NO: 5 also contains a Nde I restriction
endonuclease site (underlined) and an ATG (AUG)
methionine codon (bold) and SEQ ID NO: 6 also contains an
Xho I site. RNA was isolated from HeLa cells and first
strand cDNA was prepared and used for a template by PCR

10 using SEQ ID NOS: 5 and 6 as primers. The resulting
2.1 kilobase (kb) fragment was gel purified, ^{32}P-labeled
using oligo-dT and random primers, and used to screen a
human fetal brain library (Clontech; Palo Alto CA) under
high stringency conditions (50% formamide, 42°C; Sambrook

15 et al., *supra*, 1989).

In order to obtain the 5'-end of the cDNA
encoding IKKα, positive plaques from above were screened
by PCR using two internal primers,
(5'-CATGGCACCATCGTTCTCTG-3'; SEQ ID NO: 7), which is

20 complementary to the sequence including the Ban I site
around position 136 of SEQ ID NO: 1, and
(5'-CTCAAAGAGCTCTGGGGCCAGATAC-3'; SEQ ID NO: 8), which is
complementary to the sequence including the Sac I site
around position 475, and a vector specific primer

25 (TCCGAGATCTGGACGAGC-3'; SEQ ID NO: 9), which is
complementary to vector sequences at the 5'-end of the
cDNA insert. The longest PCR product was selected and
sequenced by the dideoxy method.

DNA sequencing revealed that the cloned IKKα

30 cDNA contained an additional 31 amino acids at the
N-terminus as compared to human CHUK. The human IKKα
shares a high amount of sequence identity with a protein
designated mouse CHUK (GenBank Accession #U12473;
Connelly and Marcu, *supra*, 1995). Although the mouse

35 CHUK contains a domain having characteristics of a

serine-threonine protein kinase, no functional activity
of the protein was reported and no potential substrates
were identified. The putative serine-threonine protein
kinase domain of human CHUK was truncated at the
5   N-terminus.

B.   Expression of human IKKα or of an antisense IKKα
     nucleic acid in a cell:

        The full length IKKα cDNA and a cDNA encoding
the Δ31 human CHUK protein (Connelly and Marcu, *supra*,
10 1995) were subcloned into the Nde I and Xho I sites of a
bacterial expression vector encoding a carboxy terminal
FLAG epitope and HIS6 tag. Mammalian cell expression
vectors were constructed by cleaving the bacterial
expression vector with Nde I and Hind III, to release the
15 cDNA inserts, converting the ends of the inserts to blunt
ends using Klenow polymerase, and ligating the cDNA
inserts encoding the full length IKKα or the Δ31 human
CHUK into pCDNA3 (Invitrogen).

        Alternatively, the IKKα cDNA and Δ31 cDNA were
20 subcloned into the Bst XI site of the pRcβactin vector
(DiDonato et al., *supra*, 1996). Orientation of the
inserts (sense or antisense) was determined by
restriction endonuclease mapping and partial sequence
using vector-specific primers. Vector containing the
25 cDNA's inserted in the sense orientation were examined
for expression of the encoded product by immunoblot
analysis using an antibody specific for the FLAG epitope.

        Transfection experiments were performed to
determine the effect of expressing the cloned IKKα in
30 HeLa cells or of expressing the cloned IKKα cDNA in the
antisense orientation. One day prior to performing the
transfections, HeLa cells were split into 35 mm dishes to
approximately 50% confluency. Cells were transfected

with 0.25 μg of a luciferase reporter gene containing an IL-8 promotor (Eckman et al., <u>Amer. Soc. Clin. Invest.</u> 96:1269-1279 (1995), which is incorporated herein by reference) along with either 1 μg pCDNA3 (Invitrogen, La
5   Jolla CA; vector control), 1 μg pRcβactin-IKKα-AA (sense orientation), 1 μg pRcβactin-IKKα-K (antisense), or 0.1 μg pCDNA-IKKα-K using the LIPOFECTAMINE method as recommended by the manufacturer (GIBCO/BRL, Gaithersburg MD).   Total DNA concentrations were kept constant by
10 addition of empty pRcβactin DNA.

Transfected cells were incubated in DMEM containing 10% FBS for 24 hr.   The cells then were washed and the growth medium was replaced with DMEM containing 0.1% FBS.   Cells either were left untreated, or were
15 treated with 20 ng/ml TNFα, 20 ng/ml IL-1α, or 100 ng/ml TPA (phorbol ester) for 3.5 hr.   Cells were harvested by scraping and washed once with PBS, then lysed in 100 μl PBS containing 1% TRITON-X100.   Luciferase assays were performed using 20 μl of lysate (DiDonato et al., *supra*,
20 1995).   The protein concentration of each extract was determined using the BIORAD protein assay kit and luciferase activity was normalized according to the protein concentrations.

NF-κB is known to induce expression for the
25 IL-8 promotor.   Thus, as expected, treatment of the vector transfected control cells with TNFα, IL-1α or TPA resulted in a 3- to 5-fold increase in normalized luciferase activity.   In comparison, in cells transfected with the cDNA encoding IKKα, treatment with TNFα, IL-1α
30 or TPA potentiated induction of luciferase activity 5- to 6-fold above the level of induction observed in the vector transfected cells.   These results indicate that expression of IKKα in cells increased the amount of NF-κB activated in response to the inducing agents.

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In cells transfected with the vector expressing the antisense IKKα nucleic acid molecule, transcription of the luciferase reporter gene induced by IL-1 or TNFα was at the limit of detection, indicating transcription

5   was almost completely inhibited due to expression of the antisense IKKα. This result indicates that the native IKKα is turned over relatively rapidly in the cells. Furthermore, treatment of the cells with the various inducing agents had no effect on the level of luciferase

10   expression of control reporter genes, which are not responsive to NF-κB, as compared to the untreated cells. Other appropriate control experiments were performed in parallel. These results demonstrate the an expression of an antisense IKKα nucleic acid molecule in a cell can

15   specifically inhibit NF-κB mediated gene expression.

EXAMPLE III

IDENTIFICATION AND CHARACTERIZATION

OF A FULL LENGTH HUMAN IKKβ SUBUNIT

This example provides methods for isolating a

20   nucleic acid molecule encoding an IKKβ catalytic subunit of IKK and characterizing the activity of the IKKβ subunit.

A.   Cloning of IKKβ cDNA:

IKKβ was purified following SDS-PAGE and

25   subjected to internal peptide sequencing (Example I). Five peptide sequences were obtained as follows: KIIDLGYAK (SEQ ID NO: 9); KXVHILN(M/Y)(V/G)(T/N/R/E)(G/N)TI(H/I/S) (SEQ ID NO: 10); KXXIQQD(T/A)GIP (SEQ ID NO: 11); KXRVIYTQL (SEQ ID

30   NO: 12); and KXEEVVSLMNEDEK (SEQ ID NO: 13), where amino acid residues that could not be unambiguously determined are indicated by an "X" and where amino acids that could not be distinguished are shown in parentheses. These

peptide sequences were used to screen the NCBI EST
database and a 336 base pair EST (EST29518; Accession No.
AA326115) encoding SEQ ID NOS: 12 and 13 was identified.
This EST was determined to correspond to amino acid
5   residues 551 to 661 of SEQ ID NO: 15.

cDNA corresponding to the EST was obtained by
PCR using first strand HeLa cDNA as a template and used
to probe a human fetal brain library (Clontech). A 1 kb
fragment was identified and used as a probe to screen a
10 plasmid based B cell library (Invitrogen). A 3 kb cDNA
insert was isolated and sequenced (Figure 2; SEQ ID
NO: 14) and encoded the full length IKKβ (SEQ ID NO: 15),
including all five proteolytic fragments (see Figure 3).

Comparison of the amino acid sequences of IKKα
15 and IKKβ revealed greater than 50% amino acid identity
(Figure 3). In addition, SEQ ID NO: 15 contains a kinase
domain, which shares 65% amino acid identity with IKKα, a
leucine zipper and a helix-loop-helix domain. Based on
the sequence homology and domain structure, the
20 polypeptide (SEQ ID NO: 15) was determined to be a member
of the IKK catalytic subunit family of proteins with IKKα
and, therefore, was designated IKKβ.

B.   Characterization of IKKβ:

This section describes the results of various
25 assays characterizing IKKβ activity, particularly with
regard to its association with IKKα. In addition,
northern blot analysis revealed that IKKβ and IKKα are
coexpressed in most tissues examined, including pancreas,
kidney, skeletal muscle, lung, placenta, brain, heart,
30 peripheral blood lymphocytes, colon, small intestine,
prostate, thymus and spleen.

1. IKKβ kinase activity

The kinase activity associated with IKKβ was
characterized using HeLa or 293 cells transiently
transfected with an HA-tagged IKKβ expression vector.
5   Transfected cells were stimulated with 20 ng/ml TNF for
10 min and HA-IKKβ was isolated by immunoprecipitation
using anti-HA antibody (Kolodziej and Young, Meth.
Enzymol. 194:508-519 (1991)). The immune complexes were
tested for the ability to phosphorylate wild type (wt)
10   and mutant forms of IκBα and IκBβ (see Example I).

Similarly to the purified IKK complex and the
complex associated with IKKα, the IKKβ immune complex
phosphorylated wt IκBα and IκBβ, but not mutants in which
the inducible phosphorylation sites (Ser-32 and Ser-36
15   for IκBα and Ser-19 and Ser-23 for IκBα) were replaced
with either alanines or threonines. However, a low level
of residual phosphorylation of full length IκBα(A32/A36)
was observed due to phosphorylation of sites in the
C-terminal portion of the protein (DiDonato et al.,
20   *supra*, 1997). Single substitution mutants, IκBα(A32) and
IκB(A36), were phosphorylated almost as efficiently as
wt IκBα, indicating that IKKβ-associated IKK activity can
phosphorylate IκBα at both Ser-32 and Ser-36.

The response of IKKβ-associated kinase activity
25   to various stimuli also was examined in HeLa cells
transiently transfected with the HA-IKKβ expression
vector. After 24 hr, the cells were stimulated with
either 10 ng/ml IL-1, 20 ng/ml TNF or 100 ng/ml TPA, then
HA-IKKβ immune complexes were isolated by
30   immunoprecipitation and IKK activity was measured. TNF
and IL-1 potently stimulated IKKβ-associated kinase
activity, whereas the response to TPA was weaker. The
kinetics of IKKβ activation by either TNF or IL-1
essentially were identical to the kinetics of activation

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of the IKKα-associated IκB kinase measured by a similar
protocol.

2. Functional interactions between IKKα and IKKβ

        As shown in Example I, IKKα and IKKβ copurified
5   in about a 1:1 ratio through several chromatographic
steps, suggesting that the two proteins interact with
each other. The ability of the IKK subunits to interact
in a functional complex and the effect of each subunit on
the activity of the other subunit was examined using
10 293 cells transfected with expression vectors encoding
Flag(M2)-IKKα or M2-IKKα and HA-IKKβ, either alone or in
combination (see Hopp et al., BioTechnology 6:1204-1210
(1988)). After 24 hr, samples of the cells were
stimulated with TNF, lysates were prepared from
15 stimulated and unstimulated cells, and one portion of the
lysates was precipitated with anti-Flag antibodies
(Eastman Kodak Co.; New Haven CT) and another portion was
precipitated with anti-HA antibodies. The IKK activity
associated with the different immune complexes and their
20 content of IKKα and IKKβ were measured.

        Considerably more basal IKK activity was
precipitated with HA-IKKβ than with Flag-IKKα. However,
the activity associated with HA-IKKβ was further elevated
upon coexpression of M2-IKKα and the low basal activity
25 associated with Flag-IKKα was strongly augmented by
coexpression of IKKβ. Immunoblot analysis revealed that
the potentiating effect of such coexpression was not due
to changes in the level of expression of IKKα or IKKβ.

        The levels of IKK activities associated with
30 IKKα and IKKβ were compared more precisely by
transfecting 293 cells with increasing amounts of HA-IKKα
or HA-IKKβ expression vectors (0.1 to 0.5 $\mu g/10^6$ cells)
and determining the kinase activities associated with the

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two proteins in cell lysates prepared before or after TNF
stimulation (20 ng/ml, 5 min); GST-IκBα(1-54) was used as
substrate. The level of expression of each protein was
determined by immunoblot analysis and used to calculate
5 the relative levels of specific IKK activity.

The HA-IKKα-associated IKK had a low level of
basal specific activity, whereas expression of HA-IKKβ
resulted in high basal specific activity that was
increased when higher amounts of HA-IKKβ were expressed.
10 However, the specific IKK activity associated with either
IKKα or IKKβ isolated from TNF-stimulated cells was very
similar and was not considerably affected by their
expression level. These results indicate that titration
of a negative regulator or formation of a constitutively
15 active IKK complex can occur due to overexpression of
IKKβ.

The ability of IKKα and IKKβ to physically
interact was examined. Immunoblot analysis demonstrated
that precipitation of HA-IKKβ using an anti-HA antibody
20 coprecipitated both endogenous IKKα and coexpressed
Flag-IKKα, as indicated by the higher amount of
coprecipitating IKKα detected after cotransfection with
Flag-IKKα. Similarly, immunoprecipitation of Flag-IKKα
with anti-Flag(M2) antibody resulted in coprecipitation
25 of cotransfected HA-IKKβ. Exposure of the cells to TNF
had no significant effect on the association of IKKα and
IKKβ.

The interaction between IKKα and IKKβ was
further examined by transfecting HeLa cells with various
30 amounts (0.1 to 1.0 μg/10^6 cells) of the HA-IKKβ vector.
After 24 hr, the cells were incubated for 5 min in the
absence or presence of 20 ng/ml TNF, then lysed. The
lysates were examined for IKK activity and for the amount
of HA-IKKβ and endogenous IKKα. Expression of increasing

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amounts of HA-IKKβ resulted in higher basal levels of IKK
activity and increasing amounts of coprecipitated IKKα.
The level of TNF stimulated IKK activity increased only
marginally in response to IKKβ overexpression and TNF had
5   no effect on the association of IKKβ and IKKα.

        Since the results described above revealed that
HA-IKKβ associates with endogenous IKKα to generate a
functional cytokine-regulated IKK complex, this
association was examined further by transfecting HeLa
10 cells with either empty expression vector or small
amounts (1 μg/60 mm plate) of either HA-IKKα or HA-IKKβ
vectors. After 24 hr, samples of the transfected cell
populations were stimulated with 20 ng/ml TNF for 5 min,
then cell lysates were prepared and separated by gel
15 filtration on a SUPEROSE 6 column. One portion of each
column fraction was immunoprecipitated with a polyclonal
antibody specific for IKKα and assayed for
IKKα-associated IKK activity, while a second portion was
precipitated with anti-HA antibody and examined for
20 HA-IKKβ- or HA-IKKα-associated IKK activity. Relative
specific activity was determined by immunoprecipitating
the complexes, separating the proteins by SDS-PAGE,
blotting the proteins onto IMOBILON membranes (Millipore;
Bedford MA), immunoblotting with anti-HA antibody and
25 quantitating the levels of IκB phosphorylation and HA-
tagged proteins by phosphoimaging. The results
demonstrated that endogenous IKKα-associated IKK activity
exists as two complexes, a larger complex of
approximately 900 kDa and a smaller one of approximately
30 300 kDa. Stimulation with TNF increased the IKK activity
of both complexes, although the extent of increase was
considerably greater for the 900 kDa complex.

        HA-IKKβ-associated IKK activity had exactly the
same distribution as the IKKα-associated activity,
35 eluting at 900 kDa and 300 kDa and, again, the extent of

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TNF responsiveness was considerably greater for the
900 kDa complex. Comparison to the IKKα-associated
activity in cells transfected with the empty vector
indicated that HA-IKKβ expression produced a modest,
approximately 2-fold increase in the relative amount of
IKK activity associated with the smaller 300 kDa complex.
These results indicate that the 300 kDa IKK complex, like
the 900 kDa complex, contains both IKKα and IKKβ.
However, the 300 kDa lacks other subunits present in the
900 kDa complex. When IKKβ was overexpressed, the
relative amount of the smaller complex increased,
indicating that some of the subunits that are unique to
the larger complex are present in a limited amount.

3. Both IKKα and IKKβ contribute to IKK activity

The relative contribution of IKKα and IKKβ to
IKK activity was examined by constructing mutant subunits
in which the lysine (K) codon present at position 44 of
each subunit was substituted with a codon for either
methionine (M) or alanine (A) codon, respectively.
Similar mutations in other protein kinases render the
enzymes defective in binding ATP and, therefore,
catalytically inactive (Taylor et al., Ann. Rev. Cell
Biol. 8:429-462 (1992)). The activity of the IKK mutants
was compared to the activity of their wild type (wt)
counterparts by cell-free translation in reticulocyte
lysates using GST-IκBα(1-54) as a substrate. Translation
of IKKα(KM) resulted in formation of IκB kinase having
only slightly less activity than the IKK formed by
translation of wt IKKα. In comparison, translation of
IKKβ(KA) did not generate IKK activity. Translation of
wt IKKβ generated IκB kinase activity as expected.

The activities of the different proteins also
was examined by transient transfection in mammalian
cells. Expression and immunoprecipitation of HA-IKKα(KM)

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resulted in isolation of cytokine stimulated IKK activity
that, after TNF stimulation, was 2-to 3-fold lower than
the activity of IKK formed by wt HA-IKKα isolated from
TNF-stimulated cells. Similarly, expression and

5   immunoprecipitation of HA-IKKβ resulted in formation of a
cytokine responsive IKK activity that, after TNF
stimulation, was 3- to 5-fold lower than the activity of
IKK generated by wt HA-IKKβ isolated from TNF stimulated
cells. In contrast to results obtained by overexpression

10 of wt HA-IKKβ, however, overexpression of HA-IKKβ(KA) did
not result in the generation of basal IKK activity.
Immunoprecipitation experiments revealed that IKKα(KM)
associates IKKβ and that IKKβ(KA) associates with IKKα
and that both IKKα and IKKβ undergo homotypic

15 interactions as efficiently as they undergo heterotypic
interactions.

Autophosphorylation of wt and kinase-defective
HA-IKKα and HA-IKKβ was examined in transiently
transfected HeLa cells. HeLa cells expressing these

20 proteins were treated with TNF for 10 min, then cell
lysates of TNF treated or untreated cells were
immunoprecipitated with HA antibodies and the immune
complexes were subjected to a phosphorylation reaction
(DiDonato et al., *supra*, 1997). Both wt HA-IKKα and

25 wt HA-IKKβ were phosphorylated and their
autophosphorylation was enhanced in TNF-stimulated
extracts. In contrast, the kinase-defective IKKα or IKKβ
mutants did not exhibit significant autophosphorylation.

4. The role of the LZ and HLH motifs in IKKα and IKKβ

30   IKKα and IKKβ both contain leucine zipper (LZ)
and helix-loop-helix (HLH) motifs, which are known to
mediate protein-protein interactions through their
hydrophobic surfaces. The role of the LZ motif in the
IKK subunit interaction was examined using an IKKα

mutant in which the L462 and L469 residues within the LZ
region were substituted with serine residues. The role
of the HLH motif was examined using an HLH mutant of IKKα
containing a substitution of L605 with arginine (R) and
5   of F606 with proline (P). The activity of the IKKα LZ⁻
and HLH⁻ mutants was examined by transient transfection in
293 cells, either alone or in the presence of
cotransfected Flag-IKKα.

         Expression of wt HA-IKKα generated substantial
10   IKK activity that was isolated by immunoprecipitation
with anti-HA, whereas very little IKK activity was
generated in cells transfected with either the
HA-IKKα(LZ)⁻ or HA-IKKα(HLH)⁻ mutant. Coexpression of the
mutant IKK subunits with Flag-IKKβ resulted in a
15   substantial increase in the IKK activity isolated by
immunoprecipitation of HA-IKKα, but had no effect on the
very low activity that coprecipitated with HA-IKKα(LZ)⁻.
However, coexpression of Flag-IKKβ did stimulate the low
level of IKK activity associated with HA-IKKα(HLH)⁻.
20   Probing of the HA immune complexes with anti-Flag(M2)
antibodies indicated that both wt HA-IKKα and
HA-IKKα(HLH)⁻ associated with similar amounts of
Flag-IKKβ, but that the HA-IKKα(LZ)⁻ mutant did not
associate with Flag-IKKβ. These results indicate that
25   the lower IκB kinase activity associated with the
IKKα(LZ)⁻ mutant is due to a defect in its ability to
interact with IKKβ. The lower IκB kinase activity of the
IKKα(HLH)⁻ mutant, on the other hand, likely is due to a
defect in the ability to interact with a second,
30   undefined protein, since the HLH mutant can interact with
IKKβ.

5. Both IKKα and IKKβ are necessary for NF-κB activation

         The contribution of IKKα and IKKβ to NF-κB
activation was examined using HeLa cells transfected with

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expression vectors encoding HA-tagged wt IKKα, IKKα(KM),
wt IKKβ and IKKβ(KA); an HA-JNK1 vector was used as a
control. NF-κB activation was assessed by examining the
subcellular distribution of RelA(p65) by indirect
5   immunofluorescence.

         HeLa cells were grown on glass cover slips in
growth medium, then transfected with 1 μg plasmid DNA by
the lipofectamine method. After 24 hr, samples of cells
were stimulated with 20 ng/ml TNF for 30 min, then
10   stimulated or unstimulated cells were washed with PBS and
fixed with 3.5% formaldehyde in PBS for 15 min at room
temperature (RT). The fixed cells were permeablized with
0.02% NP-40 in PBS for 1 min, then incubated with 100%
goat serum at 4°C for 12 hr. The cells then were washed
15   3 times with PBS and incubated with a mixture of a rabbit
anti-NF-κB p65 (RelA) antibody (1:100 dilution; Santa
Cruz Biotech) and a mouse monoclonal anti-HA antibody in
PBS containing 1% BSA and 0.2% TRITON X-100 at 37 °C for
2 hr. Cells then were washed 3 times with PBS containing
20   0.2% TRITON X-100 and incubated for 2 hr at RT with
secondary antibodies, fluorescein-conjugated goat
affinity purified anti-mouse IgG-IgM and rhodamine-
conjugated IgG fraction goat anti-rabbit IgG (1:200
dilution; Cappel). Cells were washed 4 times with PBS
25   containing 0.2% TRITON X-100, then covered with a drop of
gelvatol mounting solution and viewed and photographed
using a Zeiss Axioplan microscope equipped for
epifluorescence with the aid of fluoroscein and rhodamine
specific filters.

30   Double staining with both anti-RelA and anti-HA
revealed that expression of moderate amounts of either
wt IKKα or wt IKKβ did not produce considerable
stimulation of RelA nuclear translocation. In addition,
the wt IKK proteins did not interfere with the nuclear
35   translocation of RelA induced by TNF treatment. However,

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expression of similar levels of either IKKα(KM) or
IKKβ(KA), as determined by the intensity of the
fluorescent signal, inhibited the nuclear translocation
of RelA in TNF-treated cells. Expression of HA-JNK1 had
5   no effect on the subcellular distribution of RelA. Since
the subcellular distribution of RelA is dependent on the
state and abundance of IκB, these results indicate that
expression of either IKKα(KM) or IKKβ(KA) inhibits the
induction of IκB phosphorylation and degradation by TNF.

10   **EXAMPLE IV**

                ISOLATION OF IκB KINASE COMPLEX

            This example demonstrates a method for
    isolating the 900 kDa IκB kinase complex comprising an
    IKKα polypeptide.

15   Proteins that associate with IKKα *in vivo* were
    isolated by immunoprecipitation using HIS6 and FLAG
    epitope tags. The HIS6-FLAG-IKKα (HF-IKKα) encoding
    construct was prepared using a double stranded
    oligonucleotide, 5'-AGCTTGCGCGTATGGCTTCGGGTCATCACCATCACCA
20 TCACGGTGACTACAAGGACGACGATGACAAAGGTGACATCGAAGGTAGAGGTCA-3'
    (SEQ ID NO: 16), which encodes six histidine residues
    (HIS6), the FLAG epitope and the factor Xa site in
    tandem. The oligonucleotide was inserted using HindIII-
    NdeI site in frame with the N-terminus of the IKKα coding
25 sequence in the BLUESCRIPT KS plasmid (Stratagene;
    La Jolla CA). The HindIII-NotI fragment of this plasmid,
    which contains the HF-IKKα cDNA sequence, was subcloned
    into the pRcβactin mammalian expression vector, which
    contains a nucleic acid sequence conferring neomycin
30 resistance, to produce plasmid pRC-HF-IKKα. Expression
    of the HF-IKKα polypeptide was confirmed by western blot
    analysis using anti-FLAG antibodies.

pRC-HF-IKKα was transfected into human
embryonic kidney 293 cells and transfected cells were
selected for growth in the presence of G418. A low basal
level of IKK activity was detected in cells expressing
5   HF-IKKα and IKK activity increased several fold when the
cells were treated with TNFα. This result indicates that
the HF-IKKα expression in 293 cells is associated with
IKK activity in the cells and that such IKK activity is
inducible in response to TNFα.

10   A 293 cell line that expresses HF-IKKα was
selected and expanded to approximately 4×10^8 cells. The
cells were treated with 10 ng/ml TNFα for 5 min, then
harvested in ice cold PBS by centrifugation at 2500 x g.
The cell pellet was washed with ice cold PBS, resuspended
15   in lysis buffer (20 mM Tris, pH 7.6), 150 mM NaCl,
1% TRITON X-100, 20 mM β-glycerophosphate, 2 mM PNPP,
1 mM Na_3VO_4, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.5 mM
EGTA, 1 mM PMSF, 3 μg/ml pepstatin, 3 μg/ml leupeptin,
10 μg/ml bestatin and 25 μg/ml aprotinin), and lysed by
20   20 strokes in a glass Dounce homogenizer (pestle A).

           The homogenate was centrifuged at 15,000 rpm in
a Beckman SS34 rotor for 30 min at 4°C. The supernatant
was collected, supplemented with 20 mM imidazole and
300 mM NaCl, then mixed with 0.5 ml of a 50% slurry of
25   Ni-NTA (nickel nitrilotriacetic acid; Qiagen, Inc.;
Chatsworth CA) and stirred for 4 hr at 4°C. Following
incubation, the resin was pelleted at 200 x g and the
supernatant was removed. The resin was washed 3 times
with 50 ml binding buffer containing 25 mM imidazole.

30   Proteins bound to the resin were eluted in 2 ml
binding buffer containing 150 mM imidazole and 20 mM DTT.
The eluate was mixed with 100 μl of a 50% slurry of
anti-FLAG antibody coupled to SEPHAROSE resin using the
AMINOLINK PLUS immobilization kit (Pierce Chem. Co.;

Rockford IL) and stirred for 4 hr at 4°C. The resin was
pelleted at 1000 x g, the supernatant was removed, and
the resin was washed with 10 ml binding buffer (without
imidazole). Proteins bound to the resin then were eluted
5   with 1% SDS or with FLAG peptide and examined by 10% SDS-
PAGE.

        Silver staining revealed the presence of seven
proteins, including the HF-IKKα, which was confirmed by
western blot analysis using anti-FLAG antibody. The
10 copurified proteins had apparent molecular masses of
about 100 kDa, 63 kDa, 60 kDa, 55 kDa, 46 kDa and 29 kDa;
the endogenous 87 kDa IKKβ comigrates with the HA-IKKα
protein. These results indicate that IKKα, along with
some or all of the copurifying proteins, comprise the
15 900 kDa IκB kinase complex.

EXAMPLE V

ANTI-IKK ANTISERA

        This example provides a method of producing
anti-IKK antisera.

20   Anti-IKKα antibodies were raised in rabbits
using either His-tagged IKKα expressed in *E. coli* or the
IKKα peptide ERPPGLRPGAGGPWE (SEQ ID NO: 17) or
TIIHEAWEEQGNS (SEQ ID NO: 18) as an immunogen. Anti-IKKβ
antibodies were raised using the peptide SKVRGPVSGSPDS
25 (SEQ ID NO: 19). The peptides were conjugated to keyhole
limpet hemocyanin (Sigma Chemical Co.; St. Louis MO).
Rabbits were immunized with 250 to 500 µg conjugated
peptide in complete Freund's adjuvant. Three weeks after
the primary immunization, booster immunizations were
30 performed using 50 to 100 µg immunogen and were repeated
three times, at 3 to 4 week intervals. Rabbits were bled
one week after the final booster and antisera were

collected. Anti-IKKα antiserum was specific for IKKα and did not cross react with IKKβ.

EXAMPLE VI
USE OF AN IKK SUBUNIT IN A DRUG SCREENING ASSAY

5   This example describes an assay for screening for agents such as drugs that alter the association of an IKK subunit and a second protein that specifically associates with the IKK subunit.

             A GST-IKK subunit fusion protein or HIS6-IKK
10   subunit fusion protein can be prepared using methods as described above and purified using glutathione- or metal-chelation chromatography, respectively (Smith and Johnson, Gene 67:31-40 (1988), which is incorporated herein by reference; see, also, Example IV). The fusion
15   protein is immobilized to a solid support taking advantage of the ability of the GST protein to specifically bind glutathione or of the HIS6 peptide region to chelate a metal ion such as nickel (Ni) ion or cobalt (Co) ion (Clontech) by immobilized metal affinity
20   chromatography. Alternatively, an anti-IKK antibody can be immobilized on a matrix and the IKK-α can be allowed to bind to the antibody.

             The second protein, which can be IκB or a protein that copurifies with IKK subunit as part of the
25   900 kDa IκB kinase, for example, can be detectably labeled with a moiety such as a fluorescent molecule or a radiolabel (Hermanson, supra, 1996), then contacted in solution with the immobilized IKK subunit under conditions as described in Example I, which allow IκB to
30   specifically associate with the IKK subunit. Preferably, the reactions are performed in 96 well plates, which allow automated reading of the reactions. Various agents

such as drugs then are screened for the ability to alter
the association of the IKK subunit and IκB.

The agent and labeled IκB, for example, can be
added together to the immobilized IKK subunit, incubated
5   to allow binding, then washed to remove unbound labeled
IκB. The relative amount of binding of labeled IκB in
the absence as compared to the presence of the agent
being screened is determined by detecting the amount of
label remaining in the plate. Appropriate controls are
10   performed to account, for example, for nonspecific
binding of the labeled IκB to the matrix. Such a method
allows the identification of an agent that alter the
association of an IKK subunit and a second protein such
as IκB.

15   Alternatively, the labeled IκB or other
appropriate second protein can be added to the
immobilized IKK subunit and allowed to associate, then
the agent can be added. Such a method allows the
identification of agents that can induce the dissociation
20   of a bound complex comprising the IKK subunit and IκB.
Similarly, a screening assay of the invention can be
performed using the 900 kDa IKK complex, comprising an
IKK subunit.

Although the invention has been described with
25   reference to the examples provided above, it should be
understood that various modifications can be made without
departing from the spirit of the invention. Accordingly,
the invention is limited only by the claims.

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We claim:

1.   An isolated nucleic acid molecule,
comprising a nucleotide sequence encoding an IκB kinase
(IKK) subunit, IKKβ, which phosphorylates the inhibitor
of NF-κB (IκBα) on serine-32 and serine-36 and has an
apparent molecular mass of about 87 kiloDaltons; or a
nucleotide sequence complementary thereto.

2.   The nucleic acid molecule of claim 1,
comprising the nucleotide sequence shown in SEQ ID
NO: 14.

3.   The nucleic acid molecule of claim 1,
comprising a nucleotide sequence encoding the amino acid
sequence shown in SEQ ID NO: 15.

4.   A polynucleotide, comprising a nucleotide
sequence selected from the group consisting of:

a) a nucleotide sequence encoding at least
three contiguous amino acids of SEQ ID NO: 15;

b) a nucleotide sequence complementary to
a nucleotide sequence encoding at least three
contiguous amino acids of SEQ ID NO: 15; and

c) a mixture of nucleotide sequence,
comprising a nucleotide sequence encoding at
least three contiguous amino acids of SEQ ID
NO: 15, and a nucleotide sequence complementary
thereto, wherein said mixture comprises single
stranded polynucleotides or double stranded
polynucleotides.

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5.   An antisense nucleic acid molecule,
comprising a polynucleotide sequence complementary to at
least nine contiguous nucleotides of SEQ ID NO: 14 or
complementary to a nucleotide sequence encoding at least
5   three contiguous amino acids of SEQ ID NO: 15.

6.   An isolated nucleic acid molecule,
comprising a nucleotide sequence encoding a full length
human IκB kinase, which phosphorylates the inhibitor of
NF-κB (IκBα) on serine-32 and serine-36 and has an
10   apparent molecular mass of about 85 kiloDaltons; or a
nucleotide sequence complementary thereto.

7.   The nucleic acid molecule of claim 6,
comprising the nucleotide sequence shown in SEQ ID NO: 1.

8.   The nucleic acid molecule of claim 6,
15   comprising a nucleotide sequence encoding the amino acid
sequence shown in SEQ ID NO: 2.

9.   A polynucleotide, comprising a nucleotide
sequence selected from the group consisting of:

a)   at least nine contiguous nucleotides
20   of SEQ ID NO: 1, said at least nine contiguous
nucleotides further comprising at least three
contiguous nucleotides of the nucleotide
sequence shown as positions -35 to 92 of SEQ ID
NO: 1;

25   b)   a nucleotide sequence complementary to
said at least nine contiguous nucleotides of
SEQ ID NO: 1; and

c) a mixture of said at least nine
contiguous nucleotide and said nucleotide
sequence complementary thereto, wherein said
mixture comprises single stranded
5   polynucleotides or double stranded
polynucleotides.

10.   An antisense nucleic acid molecule,
comprising a polynucleotide sequence complementary to at
least nine contiguous nucleotides of SEQ ID NO: 1,
10   further comprising at least three contiguous nucleotides
within positions -35 to 92 of SEQ ID NO: 1, wherein said
polynucleotide sequence binds to and inhibits the
expression of a nucleic acid molecule encoding an IκB
kinase.

15   11.   A vector, comprising the nucleic acid
molecule or the polynucleotide of any of claims 1 to 10.

12.   The vector of claim 11, which is an
expression vector.

13.   The vector of claim 11, which is a viral
20   vector.

14.   A host cell containing the vector of
claim 11.

15.   An isolated IκB kinase β subunit (IKKβ),
wherein said IKKβ phosphorylates serine-32 and serine-36
25   of IκBα and has an apparent molecular mass of
87 kiloDaltons as determined by SDS-polyacrylamide gel
electrophoresis in an 8% gel under reducing conditions.

16.   The isolated IKKβ of claim 15, comprising
the amino acid sequence as shown in SEQ ID NO: 15.

17.   A peptide, comprising at least three contiguous amino acids of SEQ ID NO: 15.

18.   An isolated human IκB kinase α subunit (IKKα), wherein said IKKα phosphorylates serine-32 and serine-36 of IκBα and which has an apparent molecular mass of about 85 kiloDaltons as determined by SDS-polyacrylamide gel electrophoresis in an 8% gel under reducing conditions.

19.   The isolated human IKKα of claim 18, comprising the amino acid sequence of SEQ ID NO: 2.

20.   A peptide portion of the human IKKα of claim 19, comprising at least two contiguous amino acids within positions 1 to 31 of SEQ ID NO: 2.

21.   The peptide of claim 20, wherein one of said at least two contiguous amino acids further comprises the amino acid shown at position 30 or position 31 of SEQ ID NO: 2.

22.   An antibody that specifically binds to an epitope comprising an amino acid of SEQ ID NO: 15, provided said epitope is not present in SEQ ID NO: 2.

23.   An antibody that specifically binds to an epitope comprising an amino acid present within positions 1 to 31 of SEQ ID NO: 2.

24.   The antibody of claim 23, wherein said amino acid is the amino acid shown at position 30 of SEQ ID NO: 2.

25.   A cell line producing the antibody of any of claims 22 to 24.

26.   The cell line of claim 25, which is a hybridoma cell line.

27.   A method of identifying an agent that modulates the specific association of an IκB kinase (IKK)
5   subunit and a second protein, comprising the steps of:

a) contacting the IKK subunit and the second protein, under conditions suitable for the specific association of said IKK subunit and said second protein, with an agent
10   suspected of being able to modulate said specific association; and

b)   detecting an altered association of said IKK subunit and said second protein in the presence of said agent, wherein said altered
15   association identifies an agent that modulates the specific association of said IKK subunit and said second protein.

28.   The method of claim 27, wherein said contacting is in an *in vitro* reaction and said IKK
20   subunit is isolated.

29.   The method of claim 27, wherein said contacting is in a cell in culture.

30.   The method of claim 29, wherein said cell is selected from the group consisting of a mammalian cell
25   and a yeast cell.

31.   The method of claim 27, wherein said altered association is detected by measuring the transcriptional activity of a reporter gene.

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32.   The method of claim 27, wherein said IKK subunit is IKKα or IKKβ.

33.   The method of claim 27, wherein said second protein is an IκB protein.

5   34.   The method of claim 33, wherein said IκB protein is selected from the group consisting of IκBα and IκBβ.

35.   The method of claim 27, wherein said second protein is a subunit of a 300 kDa IKK complex or a
10   900 kDa IKK complex.

36.   The method of claim 35, wherein said second protein subunit is IKKα or IKKβ.

37.   The method of claim 27, wherein said agent is an organic molecule.

15   38.   The method of claim 27, wherein said agent is a peptide.

39.   The method of claim 38, wherein said peptide is a mutant IκB protein selected from the group consisting of a mutant IκBα containing amino acid
20   substitutions for serine-32 and for serine-36; and a mutant IκBβ containing amino acid substitutions for serine-19 and for serine-23.

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40. A method for identifying an agent that alters IκB kinase (IKK) activity, comprising the steps of:

a) incubating an isolated composition having IKK activity with an agent suspected of being able to alter said IKK activity; and

b) determining altered IKK activity of said composition in the presence of said agent, wherein said altered IKK activity identifies an agent that alters said IKK activity of said composition.

41. The method of claim 40, wherein said agent is a protein kinase inhibitor.

42. The method of claim 40, wherein said composition comprises an IKK subunit.

43. The method of claim 40, wherein said composition comprises a 300 kDa IκB kinase complex or a 900 kDa IκB kinase complex.

44. The method of claim 43, wherein said composition comprises IKKα or IKKβ.

45. A method of obtaining isolated IκB kinase (IKK) from a sample containing the IKK, comprising the steps of:

a) contacting the sample containing the IKK with an antibody the specifically binds an epitope of the IKK; and

b) obtaining isolated IKK bound to said antibody.

46.   The method of claim 45, wherein said
antibody specifically binds an IKK catalytic subunit.

47.   The method of claim 45, wherein said
catalytic subunit is IKKα or IKKβ.

48.   The method of claim 45, wherein said
antibody specifically binds a tag linked to an IKK
catalytic subunit.

49.   The method of claim 48, wherein said tag
comprises a peptide tag selected from an HA tag, a HIS6
tag and a FLAG tag.

50.   The method of claim 45, wherein said
isolated IKK is an IKK complex.

51.   The method of claim 50, wherein said IKK
complex is a 300 kDa IKK complex or a 900 kDa IKK
complex.

52.   A method of obtaining substantially
purified IκB kinase (IKK) from a sample containing the
IKK, comprising the steps of:

a) contacting the sample containing the
IKK with adenosine triphosphate (ATP)
immobilized on a matrix, under conditions
suitable for binding of said IKK with said ATP;

b) obtaining the fraction of said sample
that binds to said ATP, said fraction
containing said IKK;

c) contacting said fraction containing
said IκB kinase with IκB immobilized on a

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matrix, under conditions suitable for binding
of said IκB kinase with said IκB; and

d) obtaining substantially purified IκB
kinase from said IκB immobilized on a matrix.

5   53.   A method of modulating NF-κB activity in a
cell, comprising contacting the cell with an agent that
alters the association of an IκB kinase (IKK) or an IKK
catalytic subunit and a second protein.

54.   A method of modulating NF-κB activity in a
10   cell, comprising contacting the cell with an agent that
alters the activity of an IκB kinase.

55.   A method of modulating NF-κB activity in a
cell, comprising introducing into the cell an antisense
IκB kinase (IKK) subunit nucleic acid molecule.

15   56.   The method of claim 55, wherein said
antisense IKK subunit nucleic acid molecule is expressed
in the cell from a vector.

```
                TCGACGGAACCTGAGGCCGCTTGCCCTCCGCCCC
                                                          60
1
atggagcggccccccggggctgcggccgggcgcgggcgggccctgggagatgcgggagcgg
  M   E   R   P   P   G   L   R   P   G   A   G   G   P   W   E   M   R   E   R
61                                                       120

ctgggcaccggcggcttcgggaacgtctgtctgtaccagcatcgggaacttgatctcaaa
  L   G   T   G   G   F   G   N   V   C   L   Y   Q   H   R   E   L   D   L   K
121                                                      180
atagcaattaagtcttgtcgcctagagctaagtaccaaaaacagagaacgatggtgccat
  I   A   I   K   S   C   R   L   E   L   S   T   K   N   R   E   R   W   C   H
181                                                      240

gaaatccagattatgaagaagttgaaccatgccaatgttgtaaaggcctgtgatgttcct
  E   I   Q   I   M   K   K   L   N   H   A   N   V   V   K   A   C   D   V   P
241                                                      300

gaagaattgaatattttgattcatgatgtgcctcttctagcaatggaatactgttctgga
  E   E   L   N   I   L   I   H   D   V   P   L   L   A   M   E   Y   C   S   G
301                                                      360

ggagatctccgaaagctgctcaacaaaccagaaaattgttgtggacttaaagaaagccag
  G   D   L   R   K   L   L   N   K   P   E   N   C   C   G   L   K   E   S   Q
361                                                      420

atactttctttactaagtgatatagggtctgggattcgatatttgcatgaaaacaaaatt
  I   L   S   L   L   S   D   I   G   S   G   I   R   Y   L   H   E   N   K   I
421                                                      480

atacatcgagatctaaaacctgaaaacatagttcttcaggatgttggtggaaagataata
  I   H   R   D   L   K   P   E   N   I   V   L   Q   D   V   G   G   K   I   I
481                            (peptide 1)                540

cataaataattgatctgggatatgccaaagatgttgatcaaggaagtctgtgtacatct
  H   K   I   I   D   L   G   Y   A   K   D   V   D   Q   G   S   L   C   T   S
541                                                      600

tttgtgggaacactgcagtatctggccccagagctctttgagaataagccttacacagcc
  F   V   G   T   L   Q   Y   L   A   P   E   L   F   E   N   K   P   Y   T   A
601                                                      660

actgttgattattggagctttgggaccatggtatttgaatgtattgctggatataggcct
  T   V   D   Y   W   S   F   G   T   M   V   F   E   C   I   A   G   Y   R   P
661                                                      720

tttttgcatcatctgcagccatttacctggcatgagaagattaagaagaaggatccaaag
  F   L   H   H   L   Q   P   F   T   W   H   E   K   I   K   K   K   D   P   K
721                                                      780
tgtatatttgcatgtgaagagatgtcaggagaagttcggtttagtagccatttacctcaa
  C   I   F   A   C   E   E   M   S   G   E   V   R   F   S   S   H   L   P   Q
781                                                      840

ccaaatagcctttgtagtttaatagtagaacccatggaaaactggctacagttgatgttg
  P   N   S   L   C   S   L   I   V   E   P   M   E   N   W   L   Q   L   M   L
841                                                      900

aattgggaccctcagcagagaggaggacctgttgaccttactttgaagcagccaagatgt
  N   W   D   P   Q   Q   R   G   G   P   V   D   L   T   L   K   Q   P   R   C
901                                                      960

tttgtattaatggatcacatttttgaatttgaagatagtacacatcctaaatatgacttct
  F   V   L   M   D   H   I   L   N   L   K   I   V   H   I   L   N   M   T   S
961                                                      1020
```

FIG. 1A

```
gcaaagataatttcttttctgttaccacctgatgaaagtcttcattcactacagtctcgt
 A  K  I  I  S  F  L  L  P  P  D  E  S  L  H  S  L  Q  S  R
1021                                                              1080
attgagcgtgaaactggaataaatactggttctcaagaacttctttcagagacaggaatt
 I  E  R  E  T  G  I  N  T  G  S  Q  E  L  L  S  E  T  G  I
1081                                                              1140
tctctggatcctcggaaaccagcctctcaatgtgttctagatggagttagaggctgtgat
 S  L  D  P  R  K  P  A  S  Q  C  V  L  D  G  V  R  G  C  D
1141                                                              1200
agctatatggtttatttgtttgataaaagtaaaactgtatatgaagggccatttgcttcc
 S  Y  M  V  Y  L  F  D  K  S  K  T  V  Y  E  G  P  F  A  S
1201                                                              1260
agaagtttatctgattgtgtaaattatattgtacaggacagcaaaatacagcttccaatt
 R  S  L  S  D  C  V  N  Y  I  V  Q  D  S  K  I  Q  L  P  I
1261                                                              1320
atacagctgcgtaaagtgtgggctgaagcagtgcactatgtgtctggactaaaagaagac
 I  Q  L  R  K  V  W  A  E  A  V  H  Y  V  S  G  L  K  E  D
1321                                                              1380
tatagcaggctctttcagggacaaagggcagcaatgttaagtcttcttagatataatgct
 Y  S  R  L  F  Q  G  Q  R  A  A  M  L  S  L  L  R  Y  N  A
1381                                                              1440
aacttaacaaaaatgaagaacactttgatctcagcatcacaacaactgaaagctaaattg
 N  L  T  K  M  K  N  T  L  I  S  A  S  Q  Q  L  K  A  K  L
1441                                                              1500
gagttttttcacaaaagcattcagcttgacttggagagatacagcgagcagatgacgtat
 E  F  F  H  K  S  I  Q  L  D  L  E  R  Y  S  E  Q  M  T  Y
1501                                                              1560
gggatatcttcagaaaaaatgctaaaagcatggaaagaaatggaagaaaaggccatccac
 G  I  S  S  E  K  M  L  K  A  W  K  E  M  E  E  K  A  I  H
1561                                                              1620
tatgctgaggttggtgtcattggatacctggaggatcagattatgtctttgcatgctgaa
 Y  A  E  V  G  V  I  G  Y  L  E  D  Q  I  M  S  L  H  A  E
1621                                                              1680
atcatgggctacagaagagccccctatggaagacgtcagggagacttgatggaatctctg
 I  M  G  L  Q  K  S  P  Y  G  R  R  Q  G  D  L  M  E  S  L
1681                                                              1740
gaacagcgtgccattgatctatataagcagttaaaaacacagaccttcagatcactcctac
 E  Q  R  A  I  D  L  Y  K  Q  L  K  H  R  P  S  D  H  S  Y
1741                                                              1800
agtgacagcacagagatggtgaaaatcattgtgcacactgtgcagagtcaggaccgtgtg
 S  D  S  T  E  M  V  K  I  I  V  H  T  V  Q  S  Q  D  R  V
1801                                                              1860
ctcaaggagctgtttggtcatttgagcaagttgttgggctgtaagcagaagattattgat
 L  K  E  L  F  G  H  L  S  K  L  L  G  C  K  Q  K̲ ̲I̲ ̲I̲ ̲D̲
1861                                                (peptide 2)  1920
ctactccctaaggtggaagtggccctcagtaatatcaaagaagctgacaatactgtcatg
 L̲ ̲L̲ ̲P̲  K  V  E  V  A  L  S  N  I  K  E  A  D  N  T  V  M
1921                                                              1980
ttcatgcagggaaaaaggcagaaagaaatatggcatctccttaaaattgcctgtacacag
 F  M  Q  G  K  R  Q  K  E  I  W  H  L  L  K  I  A  C  T  Q
1981                                                              2040
agttctgcccgctcccttgtaggatccagtctagaaggtgcagtaacccctcagacatca
 S  S  A  R  S  L  V  G  S  S  L  E  G  A  V  T  P  Q  T  S
```

FIG. 1B

```
2041                                                                  2100
gcatggctgcccccgacttcagcagaacatgatcattctctgtcatgtgtggtaactcct
 A   W   L   P   P   T   S   A   E   H   D   H   S   L   S   C   V   V   T   P
2101                                                                  2160
caagatggggagacttcagcacaaatgatagaagaaaatttgaactgccttggccattta
 Q   D   G   E   T   S   A   Q   M   I   E   E   N   L   N   C   L   G   H   L
2161                                                                  2220
agcactattattcatgaggcaaatgaggaacagggcaatagtatgatgaatcttgattgg
 S   T   I   I   H   E   A   N   E   E   Q   G   N   S   M   M   N   L   D   W

agttggttaacagaatga
 S   W   L   T   E   *
2221        2238
```

FIG. 1C

```
   1   CGCGTCCCTG  CCGACAGAGT  TAGCACGACA  TCAGTATGAG  CTGGTCACCT  TCCCTGACAA

  61   CGCAGACATG  CGGGGCCTGG  GAAATGAAAG  AGCGCCTTGG  GACAGGGGGA  TTTGGAAATG

 121   TCATCCGATG  GCACAATCAG  GAAACAGGTG  AGCAGATTGC  CATCAAGCAG  TGCCGGCAGG

 181   AGCTCAGCCC  CCGGAACCGA  GAGCGGTGGT  GCCTGGAGAT  CCAGATCATG  AGAAGGCTGA

 241   CCCACCCCAA  TGTGGTGGCT  GCCCGAGATG  TCCCTGAGGG  GATGCAGAAC  TTGGCGCCCA

 301   ATGACCTGCC  CCTGCTGGCC  ATGGAGTACT  GCCAAGGAGG  AGATCTCCGG  AAGTACCTGA

 361   ACCAGTTTGA  GAACTGCTGT  GGTCTGCGGG  AAGGTGCCAT  CCTCACCTTG  CTGAGTGACA

 421   TTGCCTCTGC  GCTTAGATAC  CTTCATGAAA  ACAGAATCAT  CCATCGGGAT  CTAAAGCCAG

 481   AAAACATCGT  CCTGCAGCAA  GGAGAACAGA  GGTTAATACA  CAAAATTATT  GACCTAGGAT

 541   ATGCCAAGGA  GCTGGATCAG  GGCAGTCTTT  GCACATCATT  CGTGGGGACC  CTGCAGTACC

 601   TGGCCCCAGA  GCTACTGGAG  CAGCAGAAGT  ACACAGTGAC  CGTCGACTAC  TGGAGCTTCG

 661   GCACCCTGGC  CTTTGAGTGC  ATCACGGGCT  CCGGCCCTT   CCTCCCCAAC  TGGCAGCCCG

 721   TGCAGTGGCA  TTCAAAAGTG  CGGCAGAAGA  GTGAGGTGGA  CATTGTTGTT  AGCGAAGACT

 781   TGAATGGAAC  GGTGAAGTTT  TCAAGCTCTT  TACCCTACCC  CAATAATCTT  AACAGTGTCC

 841   TGGCTGAGCG  ACTGGAGAAG  TGGCTGCAAC  TGATGCTGAT  GTGGCACCCC  CGACAGAGGG

 901   GCACGGATCC  CACGTATGGG  CCCAATGGCT  GCTTCAAGGC  CCTGGATGAC  ATCTTAAACT

 961   TAAAGCTGGT  TCATATCTTG  AACATGGTCA  CGGGCACCAT  CCACACCTAC  CCTGTGACAG

1021   AGGATGAGAG  TCTGCAGAGC  TTGAAGGCCA  GAATCCAACA  GGACACGGGC  ATCCCAGAGG

1081   AGGACCAGGA  GCTGCTGCAG  GAAGCGGGCC  TGGCGTTGAT  CCCCGATAAG  CCTGCCACTC

1141   AGTGTATTTC  AGACGGCAAG  TTAAATGAGG  GCCACACATT  GGACATGGAT  CTTGTTTTTC

1201   TCTTTGACAA  CAGTAAAATC  ACCTATGAGA  CTCAGATCTC  CCCACGGCCC  CAACCTGAAA

1261   GTGTCAGCTG  TATCCTTCAA  GAGCCCAAGA  GGAATCTCGC  CTTCTTCCAG  CTGAGGAAGG

1321   TGTGGGGCCA  GGTCTGGCAC  AGCATCCAGA  CCCTGAAGGA  AGATTGCAAC  CGGCTGCAGC

1381   AGGGACAGCG  AGCCGCCATG  ATGAATCTCC  TCCGAAACAA   CAGCTGCCTC  TCCAAAATGA

1441   AGAATTCCAT  GGCTTCCATG  TCTCAGCAGC  TCAAGGCCAA   GTTGGATTTC  TTCAAAACCA

1501   GCATCCAGAT  TGACCTGGAG  AAGTACAGCG  AGCAAACCGA   GTTTGGGATC  ACATCAGATA

1561   AACTGCTGCT  GGCCTGGAGG  GAAATGGAGC  AGGCTGTGGA  GCTCTGTGGG  CGGGAGAACG

1621   AAGTGAAACT  CCTGGTAGAA  CGGATGATGG  CTCTGCAGAC  CGACATTGTG  GACTTACAGA

1681   GGAGCCCCAT·GGGCCGGAAG  CAGGGGGGAA  CGCTGGACGA  CCTAGAGGAG  CAAGCAAGGG
```

FIG. 2A

```
1741   AGCTGTACAG GAGACTAAGG GAAAAACCTC GAGACCAGCG  AACTGAGGGT GACAGTCAGG

1801   AAATGGTACG GCTGCTGCTT CAGGCAATTC AGAGCTTCGA  GAAGAAAGTG CGAGTGATCT

1861   ATACGCAGCT CAGTAAAACT GTGGTTTGCA AGCAGAAGGC GCTGGAACTG TTGCCCAAGG

1921   TGGAAGAGGT GGTGAGCTTA ATGAATGAGG ATGAGAAGAC TGTTGTCCGG CTGCAGGAGA

1981   AGCGGCAGAA GGAGCTCTGG AATCTCCTGA AGATTGCTTG TAGCAAGGTC CGTGGTCCTG

2041   TCAGTGGAAG CCCGGATAGC ATGAATGCCT CTCGACTTAG CCAGCCTGGG CAGCTGATGT

2101   CTCAGCCCTC CACGGCCTCC AACAGCTTAC CTGAGCCAGC CAAGAAGAGT GAAGAACTGG

2161   TGGCTGAAGC ACATAACCTC TGCACCCTGC TAGAAAATGC CATACAGGAC ACTGTGAGGG

2221   AACAAGACCA GAGTTTCACG GCCCTAGACT GGAGCTGGTT ACAGACGGAA GAAGAAGAGC

2281   ACAGCTGCCT GGAGCAGGCC TCATGATGTG GGGGGACTCG ACCCCCTGAC ATGGGGCAGC

2341   CCATAGCAGG CCTTGTGCAG TGGGGGGACT CGACCCCCTG ACATGGGGCT GCCTGGAGCA

2401   GGCCGCGTGA CGTGGGGCTG CCTGGCCGTG GCTCTCACAT GGTGGTTCCT GCTGCACTGA

2461   TGGCCCAGGG GTCTCTGGTA TCCAGATGGA GCTCTCGCTT CCTCAGCAGC TGTGACTTTC

2521   ACCCAGGACC CAGGACGCAG CCCTCCGTGG GCACTGCCGG CGCCTTGTCT GCACACTGGA

2581   GGTCCTCCAT TACAGAGGCC CAGCGCACAT CGCTGGCCCC ACAAACGTTC AGGGGTACAG

2641   CCATGGCAGC TCCTTCCTCT GCCGTGAGAA AAGTGCTTGG AGTACGGTTT GCCACACACG

2701   TGACTGGACA GTGTCCAATT CAAATCTTTC AGGGCAGAGT CCGAGCAGCG CTTGGTGACA

2761   GCCTGTCCTC TCCTGCTCTC CAAAGGCCCT GCTCCCTGTC CTCTCTCACT TTACAGCTTG

2821   TGTTTCTTCT GGATTCAGCT TCTCCTAAAC AGACAGTTTA ATTATAGTTG CGGCCTGGCC

2881   CCATCCTCAC TTCCTCTTTT TATTTCACTG CTGCTAAAAT TGTGTTTTTA C
```

FIG. 2B

```
α   MERPPGLRPGAGGPWEMRERLGTGGFGNVCLYQHRELDLKIAIKSCRLELSTKNRERWCH   60
    |.:.|:|  ...:|:|||:|||||||||||  ::.| : .|||| || |||.:||||||
β   MSWSPSLTTQTCGAWEMKERLGTGGFGNVIRWHNQETGEQIAIKQCRQELSPRNRERWCL   60

α   EIQIMKKLNHANVVKACDVPEEL.NILIHDVPLLAMEYCSGGDLRKLLNKPENCCGLKES   119
    |||||::|.|:||| | |||:: |: :|:|||||||| |||||.||. ||||||:|:
β   EIQIMRRLTHPNVVAARDVPEGMQNLAPNDLPLLAMEYCQGGDLRKYLNQFENCCGLREG   120

α   QILSLLSDIGSGIRYLHENKIIHRDLKPENIVLQDVGGKIIHKIIDLGYAKDVDQGSLCT   179
    .||.|||||:|::||||||:|||||||||||||||||||:.::.:||||||||||:||||||
β   AILTLLSDIASALRYLHENRIIHRDLKPENIVLQQGEQRLIHKIIDLGYAKELDQGSLCT   180

α   SFVGTLQYLAPELFENKPYTATVDYWSFGTMVFECIAGYRPFLHHLQPFTWHEKIKKKDP   239
    ||||||||||||:|...||.|||||||||||:.|||||.|:|||||.::||. ||.|::.|..
β   SFVGTLQYLAPELLEQQKYTVTVDYWSFGTLAFECITGFRPFLPNWQPVQWHSKVRQKSE   240

α   KCIFACEEMSGEVRFSSHLPQPNSLCSLIVEPMENWLQLMLNWDPQQRGGPVDLTLKQPR   299
     |..:|:..:.|.|:|||  || ||.| |:::.|.:|.|:||||||| |.|.|||.  | |. .
β   VDIVVSEDLNGTVKFSSSLPYPNNLNSVLAERLEKWLQLMLMWHPRQRGT..DPTYGPNG   298

α   CFVLMDHILNLKIVHILNMTSAKIISFLLPPDESLHSLQSRIERETGINTGSQELLSETG   359
    ||  :|.||||||:|||||..:.| .: : ...||||:||..||.::|||  .::||||  |.|
β   CFKALDDILNLKLVHILNMVTGTIHTYPVTEDESLQSLKARIQQDTGIPEEDQELLQEAG   358

α   ISLDPRKPASQCV....LDGVRGCDSYMVYLFDKSKTVYEGPFASRSLSDCVNYIVQDSK   415
    :.| | |||.||:    |:.:. | :|:|||.||..||..::|. .::|.:|:|:.|
β   LALIPDKPATQCISDGKLNEGHTLDMDLVFLFDNSKITYETQISPRPQPESVSCILQEPK   418

α   IQLPIIQLRKVWAEAVHYVSGLKEDYSRLFQGQRAAMLSLLRYNANLTKMKNTLISASQQ   475
    .|:::|||||||:.:. | .||||:.|| |||||||||}:.||| |.  |.||||.:  |  |||
β   RNLAFFQLRKVWGQVWHSIQTLKEDCNRLQQGQRAAMMNLLRNNSCLSKMKNSMASMSQQ   478

α   LKAKLEFFHKSIQLDLERYSEQMTYGISSEKMLKAWKEMEEKAIHYAEVGVIGYLEDQIM   535
    |||||:||..|||:|||||:||||  .:||.|:|:.| ||:|||:  .   :: .  :  .::|
β   LKAKLDFFKTSIQIDLEKYSEQTEFGITSDKLLLAWREMEQAVELCGRENEVKLLVERMM   538

α   SLHAEIMGLQKSPYGRRQGDLMESLEQRAIDLYKQLKHRPSDH.SYSDSTEMVKIIVHTV   594
    .|::.|::||:||  ||:||: ::.||:.| :||:.| :||  ||| |||.|  595
β   ALQTDIVDLQRSPMGRKQGGTLDDLEEQARELYRRLREKPRDQRTEGDSQEMVRLLLQAI   598

α   QSDRVLKELFGHLSKLLGCKIQKIIDLLPKVEVALSNIKEADNTVMFMQGKRQKEIWHLL   654
    ||  :: :: ..::|||  :.||||  ::|||||||  .:| .|.:.||: :|:||||||:|:||
β   QSFEKKVRVIYTQLSKTVVCKQKALELLPKVEEVVSLMNEDEKTVVRLQEKRQKELWNLL   658

α   KIACTQSSARSLVGSSLEGAVTPQTSAWLPPTSAEHDHSLSCVVTPQDGETSAQMIEENL   714
    ||||  |..|: |::|  :: ... |  .|.   :.|  .: |:::.|.|.|::::|.
β   KIAC..SKVRGPVSGSPDSMNASRLS...QPGQLMSQPSTASNSLPEPAKKSEELVAEAH   713

α   NCLGHLSTIIHEANEEQGNSMMNLDWSWLTE............745
    |  .|..| |::. ||:.|: .|||||||
β   NLCTLLENAIQDTVREQDQSFTALDWSWLQTEEEEHSCLEQAS 756
```

FIG. 3

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/48; C12N 9/12, 15/63, 15/54; C07K 5/06, 16/40
US CL :435/15, 194, 320.1, 252.3; 530/330, 387.9; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/15, 194, 320.1, 252.3; 530/330, 387.9; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ____ Y	DIDONATO, J. et al. Mapping Of The Inducible IκB Phosphorylation Sites That Signal Its Ubiquitination And Degradation. Mol. Cell. Biol. April 1996, Vol. 14, No. 4, pages 1295-1304, see entire document.	15-21 ____ 27-39,45-51
X ____ Y	LEE, F. S. et al. Activation Of The IκBα Kinase Complex By MEKK1, A Kinase Of The JNK Pathway. 24 January 1997, Vol. 88, pages 213-222, see entire document.	15-21 ____ 27-39,45-51
X ____ Y	TRAENCKNER, E. B-M. et al. Phosphorylation Of Human IκB-α On Serines 32 and 36 Controls IκB-α Proteolysis And NF-κB Activation In Response To Diverse Stimuli. EMBO J. 1995, Vol. 14, No. 12, pages 2876-2883, see entire document	15-21 ____ 27-39,45-51

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 APRIL 1998	0 9 JUN 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks	
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/03511

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   1-21, 27-39, 45-51

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-5, drawn to a nucleic acid encoding a IκB kinase subunit IKKβ and the corresponding antisense sequence.

Group II, claims 6-14, drawn to a nucleic acid encoding a IκB kinase, the corresponding antisense molecules, a vector and a host cell.

Group III, claims 15-17, 27-39 and 45-51, drawn to a IκB enzyme IKKβ, a method of purifying it, and a method of use comprising identifying an agent that modulates the specific association of IKKβ and a second protein.

Group IV, claims 18-21, 27-39 and 45-51, drawn to a IκB enzyme IKKα, a method of purifying it, and a method of use comprising identifying an agent that modulates the specific association of IKKα and a second protein.

Group V, claims 22 and 25-26, drawn to an antibody that binds to SEQ ID NO: 15 and a cell line producing the antibody.

Group VI, claims 23-26, drawn to an antibody that binds to SEQ ID NO: 2 and a cell line producing the antibody.

Group VII, claims 40-44, drawn to a method for identifying an agent that alters IKKα activity.

Group VIII, claims 40-44, drawn to a method for identifying an agent that alters IKKβ activity.

Group IX, claim 52, drawn to a different method of purifying IκB kinase than that of Groups III or IV.

Group X, claim 53, drawn to a method of modulating activity in a cell different than the method of Groups XI or XII.

Group XI, claim 54, drawn to a method of modulating activity in a cell different than the method of Groups X or XII.

Group XII, claims 55-56, drawn to a method of modulating activity in a cell different than the method of Group X or XI.

Note that some of the groups encompass the same claims because they are drawn to two enzymes, namely IKKα and IKKβ.

The inventions listed as Groups I-XIV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The compounds of Groups (I,II), (III,IV) and (V,VI) are completely different chemical compounds and have no relation to each other.

One method claim is included in Groups III and IV. The methods of Groups VII-XII are drawn to different embodiments that do not relate to the other groups.